

**Developing, testing and implementing novel  
molecular diagnostic tests and procedures for  
malaria at the individual and population level  
in East and West Africa**

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# Summary

Malaria is a global public health issue responsible for an estimated 219 million cases and 435'000 deaths in 2017. Although malaria control interventions have led to a significant decrease in mortality over the past decade, no further progress in reducing global malaria burden was achieved since 2016. Novel malaria control approaches, such as highly effective vaccines and improved diagnostic tools are needed to prevent a resurgence of malaria as has been seen in the 1970s.

The overall goal of this PhD thesis is to contribute to the development, validation and implementation of novel molecular diagnostic tools in malaria endemic countries targeting all human infective malaria species. This thesis was conducted within the framework of six clinical trials evaluating the PfSPZ Vaccine, an experimental malaria vaccine based on the immunization with radiation-attenuated whole, purified, cryopreserved, metabolically active sporozoites in Tanzania and Equatorial Guinea. We used controlled human malaria infection (CHMI) to assess vaccine efficacy in these cohorts. Compared to large field studies, CHMI studies allow to test various vaccine regimen and dosing in smaller, well defined cohorts in a short time period.

In part one, we aimed to closely monitor malaria parasites at the individual level by establishing a robust laboratory qPCR-platform for molecular monitoring of asexual blood stage parasitaemia. This platform was used to evaluate the efficacy of PfSPZ Vaccine by homologous CHMI in Tanzanian and Equatorial Guinean adults. Part one was structured around four manuscripts:

### **Manuscript 1-3: Safety, immunogenicity and efficacy of attenuated whole sporozoite vaccines in Tanzanian and Equatorial Guinean volunteers.**

In clinical trials conducted in malaria pre-exposed volunteers from Tanzania and Equatorial Guinea, the PfSPZ Vaccine was found to be well tolerated and safe, even in high-risk populations such as infants and HIV+ volunteers. The use of CHMI in malaria pre-exposed adults has been proven to be highly effective. These trials revealed that the degree of immune response and high level of protection seen in previous studies in malaria-naïve volunteers, was not reproducible to the same level in Tanzanian and Equato-Guinean populations.

### **Manuscript 4: Controlled Human Malaria Infections in Tanzania and Equatorial Guinea: The impact of malaria pre-exposure on diagnosis and multiplication rate of *P. falciparum* parasites**

We used the parasite multiplication rate (PMR), the fold change in number of parasites in peripheral blood over one life cycle, to characterize the impact of malaria pre-exposure on the outcome of CHMI. Compared to malaria-naïve German volunteers, the parasite's ability to replicate was significantly reduced in Tanzanian and Equato-Guinean volunteers when challenged with the identical dose, strain and route of cryopreserved, purified *P. falciparum* sporozoites. We used two different approaches to demonstrate the impact of malaria pre-exposure on the multiplication rate of *P. falciparum* parasites. First, we showed that recently drug-cleared parasitaemia from an ongoing *P. falciparum* field infection or *P. falciparum* and *P. malariae* co-infection leads to a significantly reduced PMR. Second, PMR is significantly reduced after two consecutive homologous CHMI conducted in the same volunteer at 7 months interval. This data indicates the rapid acquisition or recall of parasite growth reducing immune effector mechanisms limiting the growth of asexual blood stages *in vivo*.



In part two, we aimed to adapt and extend molecular diagnostic tools for improvement of malaria surveillance at the population level by developing novel high-throughput, field-deployable and highly sensitive tools and approaches. Part two was structured around five manuscripts:

**Manuscript 5: A multiplex qPCR approach for detection of *pfhrp2* and *pfhrp3* gene deletions in multiple strain infections of *Plasmodium falciparum***

In response to the emerging threat of *P. falciparum* strains lacking *pfhrp2* and/or *pfhrp3* genes and therefore evading detection by PfHRP2-based RDTs, we developed a qPCR-based assay well suited for high-throughput identification of *pfhrp2* and *pfhrp3* gene deletions in *P. falciparum* isolates. The ability to detect “masked” *pfhrp2/3* deletions is probably the most interesting feature of our approach, because it will allow to study the epidemiology of *pfhrp2/3* deletions in malaria endemic regions where a high proportion of the population carries multiple strain *P. falciparum* co-infections.

**Manuscript 6: High Throughput Extraction and Analysis of Nucleic Acids from Rapid Diagnostic Tests for Molecular Surveillance of Malaria**

This manuscript describes our attempt to improve extraction of nucleic acids from used malaria RDTs. We used an approach which enabled us to extract total nucleic acids, including DNA and RNA from these RDT strips. This approach increased the overall sensitivity for the detection of *Plasmodium* spp. parasites in the small volume of blood which is available on an RDT stored at room temperature. We used samples collected during CHMI as a platform to evaluate and compare the ability of our protocol to detect and quantify *P. falciparum* parasites with microscopy and qPCR.

## **Manuscript 7: ELIMU-MDx: A Web-Based, Open-Source Platform for Storage, Management and Analysis of Diagnostic qPCR Data**

We developed a web-based and open-source software for storage, management and analysis of diagnostic qPCR data. In response to the vast amount of qPCR data generated during a series of CHMIs and other epidemiological studies conducted in Tanzania and Equatorial Guinea, we decided to design and build this platform which facilitates the larger scale analysis and interpretation of diagnostic qPCR data.

## **Manuscript 8: Molecular monitoring of the diversity of human pathogenic malaria species in blood donations on Bioko Island, Equatorial Guinea**

In collaboration with the central blood bank in Malabo, Equatorial Guinea we analysed 200 blood donations for the presence of *Plasmodium* spp. parasites by a novel, multiplex qPCR monitoring all human infective malaria species in a single reaction. We found that more than one quarter of the blood donations contained malaria parasites and that 75% of *P. falciparum* and 100% of *P. malariae* and *P. ovale* spp. infections were missed by routinely performed RDT and microscopy.

## **Manuscript 9: Two cases of long-lasting, sub-microscopic *Plasmodium malariae* infections in adults from Coastal Tanzania**

In this report we describe two cases of *P. malariae* infections that were identified during a study evaluating the safety, tolerability and efficacy of the PfSPZ Vaccine in Bagamoyo, Tanzania. Since these two adult participants were enrolled into a clinical trial, we were provided with a unique opportunity to study clinical manifestations of *P. malariae* over a follow up period of four months.

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# List of Abbreviations

ACT	Artemisinin-based combination therapy	MIS	Malaria indicator survey
CHMI	Controlled human malaria infection	MVIP	Malaria vaccine implementation program
DNA	Deoxyribonucleic acid	NAI	Naturally acquired immunity
DVI	Direct venous inoculation	NAT	Nucleic acid amplification technique
EG	Equatorial Guinea	PfHRP2	<i>P. falciparum</i> histidine rich protein 2
EGMVI	Equatorial Guinea Malaria Vaccine Initiative	PfSPZ	<i>P. falciparum</i> sporozoites
ELIMU	Electronic lab information & management utensil	PMR	Parasite multiplication rate
ENAR	Extraction of nucleic acids from RDTs	qPCR	Quantitative polymerase chain reaction
FDA	Food and drug administration	RAS	Radiation-attenuated sporozoites
GAP	Genetically attenuated parasites	RBC	Red blood cell
GMP	Good manufacturing practice	RDT	Rapid diagnostic test
HIV	Human immunodeficiency virus	RNA	Ribonucleic acid
IHI	Ifakara Health Institute	RT-qPCR	Reverse transcription qPCR
IRS	Indoor residual spraying	TBS	Thick blood smear
ITN	Insecticide-treated net	TZ	Tanzania
LAMP	Loop mediated isothermal amplification	VE	Vaccine efficacy
LOD	Limit of detection	WHO	World Health Organization

# Chapter I

## Introduction

## 1.1 Malaria – An overview

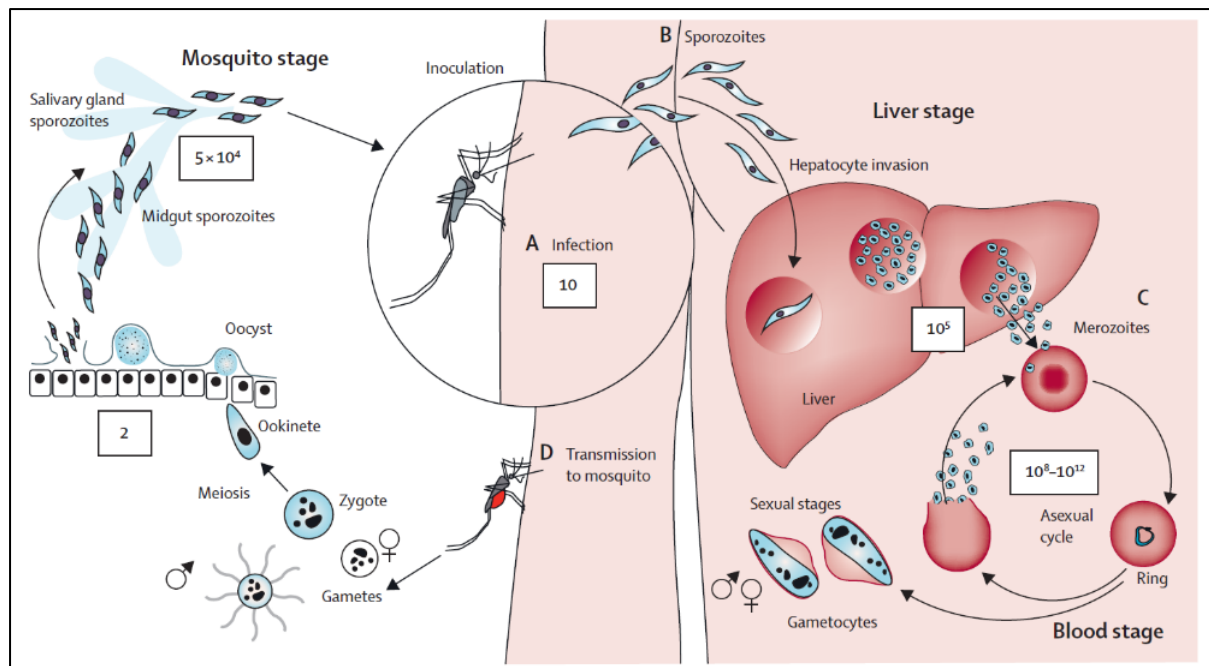
### 1.1.1 *Plasmodium* spp. life cycle

Six species of the genus *Plasmodium* are known to infect humans, namely *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri* and *P. knowlesi* [1]. The description of the life cycle of these parasites is crucial for our understanding of malaria and how intervention tools can break the transmission cycle. The basic principles are known for more than 100 years, when Ronald Ross discovered that malaria is transmitted by mosquitoes and Charles Laveran linked the *Plasmodium* parasites to malaria disease. Both of these important milestones were later awarded with the Nobel Prize [2, 3].

Malaria is a vector-borne disease that is transmitted to humans by *Plasmodium*-infected female *Anopheles* mosquitoes during a blood meal. Successful malaria transmission relies on an interaction between parasite, mosquito and human host (Figure 1). The transmission cycle starts with the inoculation of an estimated 1-100 sporozoites by a mosquito into the human host [4]. The sporozoites migrate through the skin, where they spend the majority of their extracellular time, and use the bloodstream to reach liver, where they infect hepatocytes within minutes [5]. The asymptomatic liver stage lasts one to two weeks, depending on species, strain and the hosts' immunity [1]. After asexual replication inside hepatocytes, ten thousands of merozoites are released into the bloodstream [6]. *P. vivax* and *P. ovale* spp. are able to form dormant liver-stages, named hypnozoites, which can be reactivated and cause malaria relapse [7]. After entering the bloodstream, the parasites enter the erythrocytes within seconds to minutes. This quick disappearance from the circulation reduces the risk of being exposed to the host immune system [8]. Once the merozoites have invaded erythrocytes, the



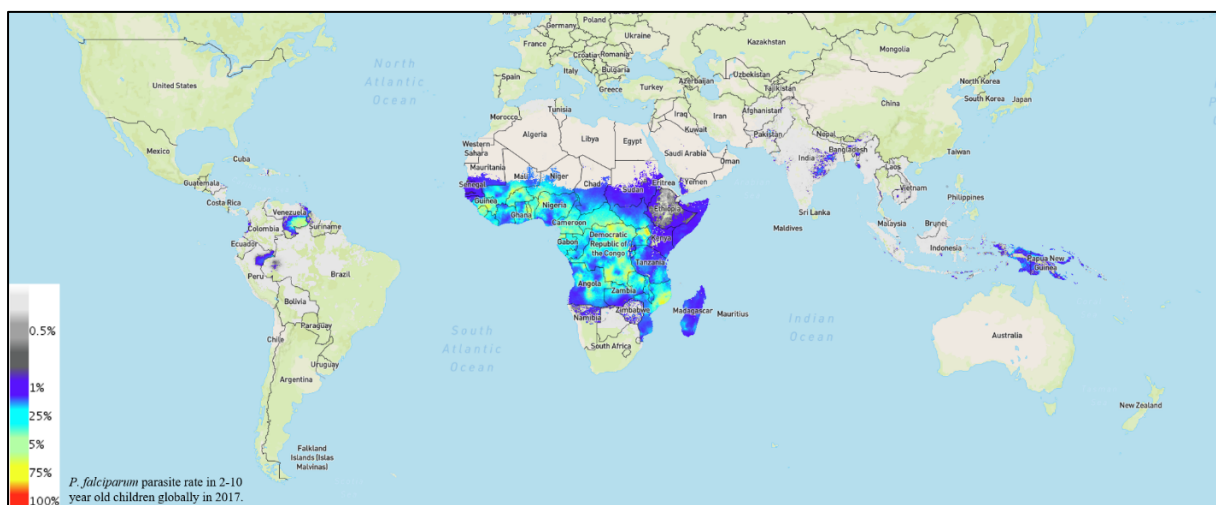
asexual blood-stage cycle starts with the ring stage that evolves into a trophozoite and finally into a schizont. As for *P. falciparum*, during one cycle, each merozoite passing through the ring, trophozoite, and schizont stages, grows and divides into 8–32 fresh merozoites until the erythrocyte ruptures and merozoites are released [9]. This cycle is repeated over many times and leads to clinical symptoms like fever each time parasites break free from the schizont stages to invade new erythrocytes [10]. One asexual blood-stage cycle takes roughly 48 hours for *P. falciparum*, *P. vivax* and *P. ovale* spp., while it takes 72 hours for *P. malariae* and 24 hours for *P. knowlesi* [11]. A small proportion of blood-stage parasites develop into gametocytes, the sexual stage of *Plasmodium* spp., which is transmitted to mosquitoes. Inside the mosquito gut, sexual reproduction takes place, when the gametocytes form an ookinete which develops into an oocyst. The oocyst bursts and liberates sporozoites, which migrate to the salivary glands to re-start the transmission cycle with the next blood meal. The entire cycle, including the mosquito, liver and blood stage, takes on average one month [11].



**Figure 1. The life cycle of *Plasmodium* spp. parasites.** The life cycle can be divided into three stages, the mosquito, the liver and the blood-stage. (A) The cycle starts with the inoculation of sporozoites by female *Anopheles* mosquitoes. (B) The sporozoites invade hepatocytes where they develop into merozoites. (C) The merozoites are then released into the blood where they invade erythrocytes. The asexual blood-stage cycle consists of an exponential parasite replication phase causing malaria-related symptoms. (D) A proportion of blood-stage parasites develop into gametocytes, which are then transmitted to mosquitoes. Inside the mosquito the gametes fuse to zygotes and develop step-by-step into transmissible sporozoites. Figure adapted from White *et al.* [11]

### 1.1.2 Epidemiology and burden of malaria

Malaria is a global public health issue with an estimated 219 million cases resulting in 435 000 deaths in 2017. More than 90% of these malaria cases and deaths occurred in Sub-Saharan Africa with Nigeria (25%), Democratic Republic of the Congo (11%), Mozambique (5%) and Uganda (4%) carrying almost half of the global burden. *P. falciparum* is the most pathogenic malaria species accounting for the vast majority of malaria cases and deaths [12]. Malaria endemicity is restricted to geographical areas where *Anopheles* mosquitoes can be found (Figure 2). The *P. falciparum* parasite rate, the proportion of the population found to carry asexual blood-stage parasites in their peripheral blood, is used to characterize the malaria risk and transmission intensity [13, 14].



**Figure 2. *P. falciparum* endemicity in 2017.** The Malaria Atlas Project (MAP, <https://map.ox.ac.uk/>) was used to map the *P. falciparum* parasite rate, in 2-10 year old children with data collected in 2017.

Clinically, malaria can present as uncomplicated or severe malaria. Symptoms of uncomplicated malaria are usually unspecific which makes clinical diagnosis unreliable [1]. The manifestations of severe malaria often include severe anaemia and/or coma in case of cerebral malaria [11]. Other common manifestations of severe malaria include acute lung injuries, which can progress to acute respiratory distress syndrome and acute kidney injuries [1]. The case fatality rate of treated cerebral malaria is usually 10 - 20% [1], while when treated, uncomplicated malaria has a mortality of roughly 0.1% [11]. In 2017, there were estimated 435'000 deaths from malaria globally with children under five years as the most vulnerable group affected by malaria. They accounted for 61% of all malaria deaths worldwide [12]. Women pregnant for the first time are at great risk too. These women have higher risks of severe malaria and death than their nonpregnant counterparts [15]. It is estimated that twenty-five million pregnant women are currently at risk for malaria, which accounts for over 10,000 maternal and 200,000 neonatal deaths per year [16]. Beside children under the age of five and pregnant women during their first pregnancy, other high-risk populations include persons with human immunodeficiency virus (HIV) infection and nonimmune travellers [10]. These vulnerable groups have in common that their immune system lacks the ability to control parasitaemia during a malaria infection. Through repeated exposure to malaria parasites, individuals living in malaria endemic regions develop naturally-acquired immunity (NAI) resulting in the ability to control blood-stage parasite multiplication [17]. These asymptomatic individuals can account for the majority of infections in the population [18, 19]. Beside these fatal outcomes, malaria has also severe socio-economic consequences. Countries with intensive malaria transmission have significantly reduced economic growth, 10% reduction in malaria is projected with 0.3% higher economic growth [20]. Malaria has a negative impact on development, investment, worker productivity, absenteeism, premature mortality and medical costs [21].

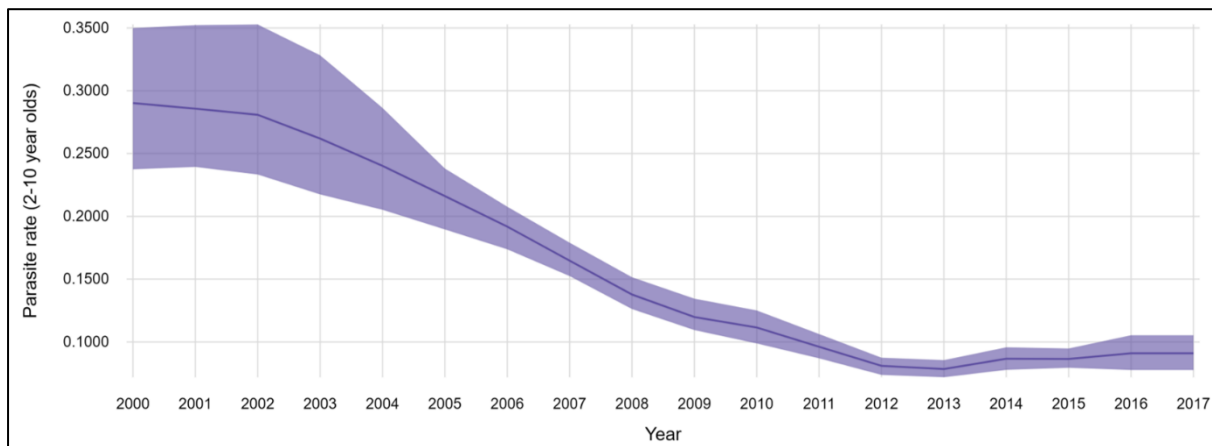
### 1.1.3 *Plasmodium malariae* and *ovale* spp. - the neglected malaria parasites

*P. malariae* is widespread throughout all major malaria-endemic regions worldwide [22], while *P. ovale* spp. is found mainly in Africa and southeast Asia [23]. The prevalence of *P. malariae* and *P. ovale* spp. in the affected population is thought to be underestimated due to the difficulties in detection. Microscopic diagnosis of *P. malariae* asexual blood-stage parasites is hampered by the usually low parasitaemia and morphological similarities to *P. falciparum* [24], and *P. ovale* spp. has the ability to form hypnozoites and therefore is not detectable in peripheral blood [25]. Lack of sensitive *P. malariae* and *P. ovale* spp. diagnosis methods applicable in the field and the research focus on *P. falciparum* has resulted in significant knowledge gaps regarding the potential spectrum of clinical manifestations and burden of *P. malariae* and *P. ovale* spp. infections [23].

Clinical episodes of *P. malariae* infections are characterized by a mild illness caused by low numbers of parasites which can persist for extremely long periods, often for years [22]. There are reports of cases of *P. malariae* caused illness decades after malaria eradication from these regions [26, 27]. Incidences of *P. ovale* spp. episodes are low [23] but relapse can occur up to 4 years after infection [25]. Using whole genome sequencing, it was demonstrated that *P. ovale* spp. essentially consists of two distinct species termed *P. ovale curtisi* and *P. ovale wallikeri* [28]. Recently, a study conducted in Gabon described different infection dynamics of *P. ovale curtisi* compared to *P. ovale wallikeri* [29]. Despite the relatively mild disease caused by *P. malariae* and *P. ovale* spp. compared to *P. falciparum*, both species should be included in future research agendas. The health burden of such chronic or re-occurring infections in an endemic context is not clear and little is known about the potential interactions caused by co-infections with different *Plasmodium* spp. species [23].

#### 1.1.4 Malaria control methods

Current strategies to fight malaria include vector control using insecticide-treated mosquito nets (ITNs) and indoor residual spraying with insecticides (IRS), as well as access to diagnostic testing and antimalarial treatment. The combination of these tools has contributed to a reduction of 20 million malaria cases globally since 2010 [12]. Exemplary for this trend, the *P. falciparum* parasite rate in Tanzanian children obtained from 2000 to 2017 is shown (Figure 3).



**Figure 3.** *P. falciparum* parasite rate in Tanzania from 2000-2017. The Malaria Atlas Project (MAP, <https://map.ox.ac.uk/>) was used to plot the *P. falciparum* parasite rate, in 2-10 years old children based on data collected between 2000 and 2017.

The first pillar of successful malaria control programs is an effective case management of patients with malaria, which includes rapid diagnosis and prompt treatment. Rapid diagnostic tests (RDTs) provide test results within 20 minutes [30] and in Sub-Saharan Africa, RDTs have almost completely replaced light microscopy for malaria diagnosis [12]. The treatment

of malaria was revolutionised by the introduction of the artemisinin derivatives and their drug combinations, which are rapidly effective, safe, and well tolerated [31]. With the widespread resistance to chloroquine and sulfadoxine–pyrimethamine, artemisinin-based combination treatments (ACTs) are now the recommended first line treatment for malaria [1].

The second pillar is vector control, ITNs [32] have contributed substantially to the recent reduction in malaria morbidity and mortality [33]. Additionally, IRS with insecticides that persist and kill mosquitoes has also proven to be an important component of malaria control [34].

Alarmingly, for the second year since 2016, there is a flat-lining of what had been a steady decline in the global malaria epidemic [12, 35]. Figure 3 demonstrates this trend in the epidemiological context of Tanzania. The global malaria control efforts, especially among high-burden countries, are under attack by reduced international funding. According to the WHO, the average level of funding per person at risk declined between 2015–2017 compared to 2012–2014 by 20% [12]. Additionally, there is an increased emergence of drug and insecticide resistance of the parasite and vector, respectively, observed. An additional 116,000 malaria deaths annually is predicted under the scenario of widespread artemisinin resistance [36]. The extensive use of insecticides for ITNs and IRS resulted in a dramatic increase of resistant mosquito populations in Africa, presumably reflecting the increased selection pressure [37]. Still, ITNs continue to be an effective tool for malaria prevention, even in areas where mosquitoes have developed resistance to pyrethroids, the only insecticide class currently used in ITNs [12]. Facing these challenges, besides an increase in funding for malaria control programs, the implementation of alternative intervention tools would ensure the sustainability of malaria control.

### 1.1.5 Malaria diagnosis

The rapid and accurate diagnosis of malaria cases is an essential factor in the control of malaria. Diagnosis of malaria is performed in blood collected from potentially infected humans. Currently, three different major diagnostic approaches are used for malaria diagnosis, including the direct visualization of blood-stage parasites by microscopy, detection of parasite-derived antigens using RDTs and amplification of parasite-specific nucleic acids by nucleic acid amplification techniques (NATs).

The gold standard for malaria diagnosis remains light microscopy of Giemsa stained blood smears. Thick blood smears (TBS) provide sensitivity and allow quantitation of infection. Thin blood smears allow the identification of different malaria species [38], which can be difficult for some species based on the similar morphology of the trophozoite stage [24]. Identification of blood stage parasites is time consuming and labour intensive and the lack of skilled technologists leads to poor quality [39]. A study which assessed the performance of malaria microscopy in the context of a clinical trial concluded that diagnostic errors occur with microscopy, including false positive findings, and that these errors can affect clinical trial results [40].

RDTs are nowadays widely used since they offer less dependence on the availability of laboratory infrastructure, and can be employed by inexperienced health care workers in resource limited settings where electricity might be a shortage [30]. Malaria RDTs are an immune-chromatographic assay based on a lateral-flow device. They detect circulating malaria antigens in small amounts of capillary blood, usually 5 to 15  $\mu$ L, using antigen specific antibodies [24]. The majority of antibodies in use recognise the *P. falciparum*-derived histidine-rich protein 2 (PfHRP2) [39]. The low costs, rapid testing, wide availability and simplicity in use and interpretation makes RDTs highly valuable to national malaria control programs.



This is reflected in 245 million RDTs distributed worldwide and an estimated 75% of malaria tests conducted in Sub-Saharan Africa being based on RDTs in 2017 [12]. However, RDTs have several inherent limitations that reduce their usefulness for epidemiological studies of malaria. Since PfHRP2 is only expressed by *P. falciparum*, the other human infective malaria species will be missed by these RDTs. Alternative antibodies targeting Pan-*Plasmodium* antigens such as lactate dehydrogenase (pLDH) or aldolase, are on the market. However, these RDTs have a ten-fold lower sensitivity compared to PfHRP2-based RDTs [38, 39]. *P. malariae* and *P. ovale* spp. infections, which are often found to occur co-endemic in areas together with *P. falciparum* are usually characterized by very low parasitaemia in peripheral blood [23]. These non-*falciparum* *Plasmodium* species will hardly be detected by malaria surveillance programs depending on RDTs only.

An increasing number of reports are raising the issue of *pfhrp2/pfhrp3* gene deletion in circulating *P. falciparum* populations resulting in false negative PfHRP2-based RDTs - even at high parasite densities. These deletions are found around the world, with different proportions of the local parasite population affected. Among the areas with the highest proportions of reported *pfhrp2* deletions are South America followed by Sub-Saharan Africa [41]. Since malaria control programmes depend on reliable diagnosis of malaria cases using RDTs, parasites lacking *pfhrp2/3* genes pose a threat to malaria control programs [42].

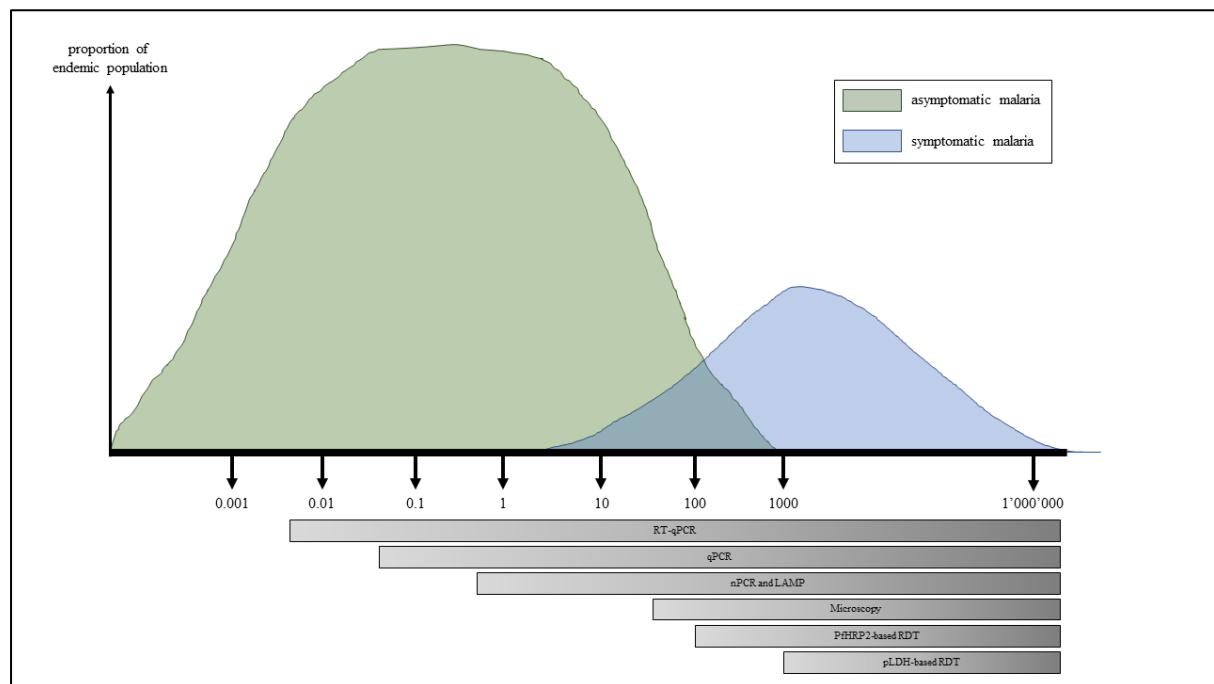
NATs are more expensive and require advanced laboratory infrastructure but provide much better sensitivity and broader diagnostic capabilities. NATs, such as polymerase chain reaction (PCR), use amplified parasite-specific nucleic acids as a biomarker for infection with *P. falciparum* parasites [43]. These techniques are also easily expandable to non-*falciparum* *Plasmodium* species [44]. Therefore, NAT-based assays for detection, quantification and identification of malaria parasites add substantially more information to malaria surveillance programs [45].

The distribution and detection of the *P. falciparum* infection reservoir found in populations living in endemic regions is illustrated in Figure 4. Clinical symptoms are strongly associated with high parasite density and usually parasitaemia levels below 100 parasites/ $\mu$ L are asymptomatic, particularly in semi-immune populations [1, 46].

Microscopy and RDTs are critical diagnostic tools for identifying symptomatic malaria infections enabling appropriate treatment. However, due to the reduced performance in infections with low parasite density, their use for diagnosis of infection in asymptomatic individuals is rather limited [17]. It is a well-established fact that the limit of detection (LOD) of microscopy conducted by an expert reader is 20 – 50 parasites/ $\mu$ L, but for non-experienced microscopists, it is up to ten-fold higher [38]. LOD of standard PfHRP2-based RDTs is about 100 parasites/ $\mu$ L, while LDH-based RDTs detect *Plasmodium spp.* infections with a LOD of about 1000 parasites/ $\mu$ L [47–49]. Novel “ultra-sensitive” RDTs were recently introduced, which have a slightly better sensitivity for *P. falciparum*, but still fail to detect a substantial proportion of parasite carriers [50] and there is probably no advantage over conventional RDTs for diagnosis of febrile patients [51].

As already mentioned above, for the detection of *Plasmodium spp.* parasites, NATs have proven to perform better than microscopy or RDTs. The parasites’ multi-copy 18S small subunit ribosomal deoxyribonucleic acid (rDNA) and/or its highly expressed ribonucleic acid (rRNA) is the most frequently used biomarker for NATs [52] and recently received qualification through the US Food and Drug Administration as a malaria biomarker for use in CHMI studies at non-endemic sites [53]. Different methods for 18S rDNA/rRNA detection are currently in use with a great variation in sensitivity. Qualitative methods targeting 18S rDNA, such as nested PCR [54] or LAMP [55], reach LODs of 0.1-10 parasites/ $\mu$ L. The use of quantitative PCR (qPCR) [56], does not only allow absolute quantification of infections but also lowers the LOD to less than 1 parasite/ $\mu$ L. The use of reverse transcription qPCR (RT-

qPCR), amplifying total nucleic acids (RNA and DNA) of the 18S genes [57–59], is further increasing sensitivities of NATs. Alternatively, qPCR assays targeting multi-copy genomic sequences are able to achieve sensitivities close to 18S rRNA-based RT-qPCR assays [60].



**Figure 4. *P. falciparum* infection reservoir in endemic populations.** Typical parasite infection densities for asymptomatic and symptomatic populations living in endemic regions are shown. The dynamic ranges of different diagnostic tools are indicated. The creation of this figure was inspired by Manning *et al.* [61]

## 1.2 Host immune response and development of vaccines

### 1.2.1 Naturally acquired immunity to malaria

In malaria endemic regions, severe disease and the resulting deaths are mostly found among in children below the age of five years [1]. This vulnerable population lacks NAI, which is thought to develop during repetitive exposure to malaria parasites [17, 62]. NAI mediates protection against clinical disease but not re-infection, resulting in a high proportion of the population carrying asymptomatic infections at low parasite density [18, 19]. The mechanism is thought to be mediated by a broad range of antibodies directed against blood-stage antigens and therefore limiting parasite replication [46, 63]. These antibodies are involved in blocking merozoite invasion of erythrocytes, opsonize merozoites and infected erythrocytes for phagocytosis or fix and activate complement on the merozoite surface resulting in parasite lysis [64]. Although the mechanisms of NAI are poorly understood, it had been shown that it is highly effective. Cohen *et al* showed 1961 in a proof of concept study that transfer of immunoglobulins from malaria immune adults to children with clinical malaria reduced parasite density and subsequently also clinical symptoms [65]. NAI provides solid protection against severe malaria and death reflected in the fact that most malaria episodes are reported in populations lacking NAI. Older children and adults constantly exposed to malaria rarely experience life threatening complications caused by malaria [46].

### 1.2.2 Immunological correlates of vaccine efficacy

Understanding immunity to malaria becomes increasingly important in development of malaria vaccines. The identification of immunological correlates of protection could possibly lead to improved vaccine designs or immunization regimens. Immunological correlates could also reduce costs and complexity of clinical trials, especially when exposure to malaria is low, if used as a surrogate marker for protection [63]. Given the complexity of the interaction between the malaria parasite and the host, it is not surprising that no clear immunological correlates of malaria protection have been identified so far [10]. Complexity of the parasite, including its life cycle, strain diversity and antigenic variation, as well as host-derived factors such as different levels of pre-existing immunity, genetics and co-infections with other pathogens may hamper the identification of immunological markers associated with malaria in field trials. Therefore, CHMI studies might provide a promising alternative platform to identify correlates of protection [63, 66].

### 1.2.3 Malaria vaccines

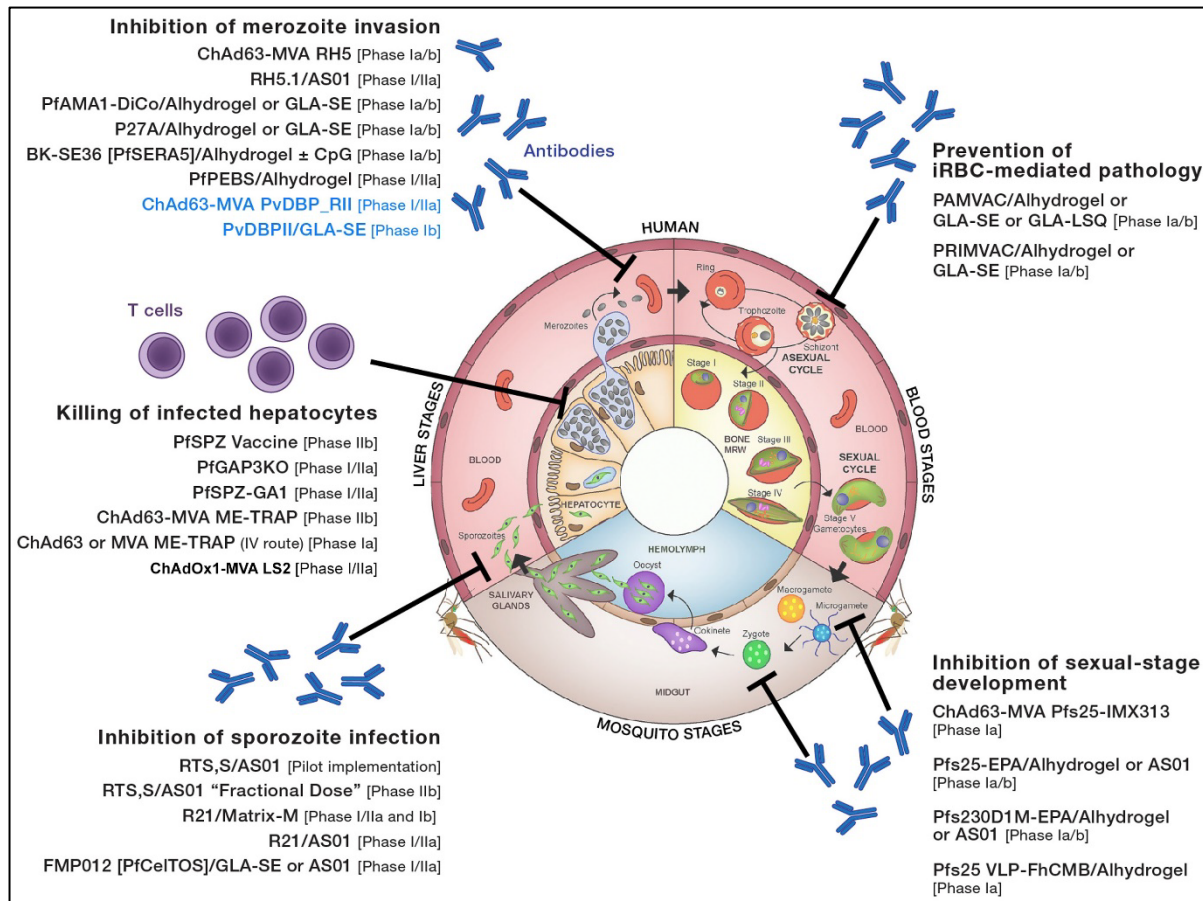
The introduction of an effective malaria vaccine, complementary to existing intervention tools, would be a game-changing milestone on the road towards malaria elimination. There are numerous potential malaria vaccine candidates under evaluation in clinical trials. Currently investigated vaccine candidates, their efficacy and mode-of-action are illustrated in Figure 5 and have been extensively reviewed recently [10, 63, 67–69].

Depending on which stage of the parasite's life cycle is being targeted, these vaccine candidates can be divided into pre-erythrocytic, blood-stage and transmission-blocking vaccines.

Successful pre-erythrocytic vaccines would lead to a sterile immunity by preventing the development of blood-stage parasitaemia with the consequence of obviating clinical symptoms and blocking transmission. Pre-erythrocytic immunity could be achieved by preventing hepatocyte invasion via antibodies that target sporozoites or *P. falciparum*-specific CD8 T-cells that target infected hepatocytes [10]. The two most advanced pre-erythrocytic vaccine candidates, RTS,S/A01 and PfSPZ Vaccine are discussed throughout this thesis extensively. On the contrary, blood-stage vaccines mimic NAI, by inducing a strong humoral immune response against target proteins expressed on the surface of infected erythrocytes or involved in merozoite invasion or egress [10]. A blood-stage vaccine, targeting the apical membrane antigen 1 (AMA1), showed high immunogenicity in malaria-exposed adults [70], but failed to provide significant protection against clinical malaria in a field trial in Mali [71]. Different subunit vaccines based on three merozoite surface proteins (MSP1, MSP2 and MSP3) were tested in different malaria pre-exposed populations [72, 73]. GMZ2 is a fusion protein of fragments of glutamate-rich protein (GLURP) and MSP3, which showed 14% vaccine efficacy against clinical malaria in a phase 2 trial in children from Burkina Faso, Gabon, Ghana and Uganda [74]. Among candidates earlier in the clinical evaluation phase are P27A and RH5. The candidate vaccine P27A has been considered safe and induced a particularly robust immunogenic response in combination with GLA-SE adjuvant in Swiss and Tanzanian volunteers [75]. Currently, the safety and immunogenicity of ChAd63 RH5 and MVA RH5 in adults, children and infants is tested in Bagamoyo, Tanzania (Ally Olotu, personal communication, Clinical trial registration: NCT03435874).

A third group of vaccine candidates aim to prevent transmission by targeting the development of gametocytes or inhibiting parasite development in mosquitoes. Transmission-blocking vaccines do not provide an direct benefit to the vaccinated individual but would contribute to a reduction of malaria infections in the population [76]. In a Malian field trial, the safety and

functional immunogenicity of Pfs25H-EPA/Alhydrogel was assessed. Although, Pfs25H-EPA/Alhydrogel was well tolerated, the number of volunteers who infected at least one mosquito by direct skin feeds did not differ between recipients of this transmission blocking vaccine after the fourth dose and a comparator vaccine (Euvax B, recombinant hepatitis B vaccine) [77].



**Figure 5. Vaccine candidates targeting different stages of the *P. falciparum* life cycle.** Pre-erythrocytic vaccines inhibit sporozoite infection or kill infected hepatocytes. Blood-stage vaccines target blood-stage antigens and induce humoral immune response to inhibit merozoite invasion or parasite replication. Transmission-blocking vaccines inhibit sexual-stage development. Figure adapted from Draper *et al* [67].

Despite the need for a vaccine and decades spent on research and development, no vaccine against malaria has been licensed until today. Several obstacles to malaria vaccines have been identified. The complex life cycle, genetic diversity and antigenic variation are among the parasite-derived challenges [78]. Additional impediments are found in the human host. The required immune responsiveness of the target population can be compromised by nutritional status, different levels of malaria pre-exposure or co-infections [63].

Nevertheless, promising advances have been made in the past decade. The large-scale pilot implementation program of the currently most advanced malaria vaccine candidate - RTS,S/AS01- in three African countries has started in April 2019 [79]. This hybrid recombinant subunit vaccine, consisting of the central tandem repeat and carboxyl terminal portion of *P. falciparum* circumsporozoite protein (PfCSP) fused to the hepatitis B surface antigen, acts as a pre-erythrocytic stage vaccine. The antigen is administered with AS01, a liposome-based vaccine adjuvant system containing two immune-stimulants: 3-O-desacyl-4'-monophosphoryl lipid A and the saponin QS-21 [80]. In a large phase 3 clinical trial conducted from 2009 to 2012 and including more than 15'000 infants and children from seven Sub-Saharan African countries, RTS,S/AS01 vaccine efficacy (VE) against clinical malaria was found to be 36.3% for children aged 5-17 months and 25.9% for infants aged 6-12 weeks at first vaccination during a follow up period of 12 months after the last dose [81]. A declining VE over time was described by Olotu *et al.*; over a 4-year period VE was only 16.8% in Tanzanian and Kenyan children aged 5-17 months [82]. However, the RTS,S vaccine has set the benchmark against which other vaccination approaches will be compared in future studies.



#### 1.2.4 Whole sporozoite vaccines

An alternative approach to subunit vaccines is the immunization with live, attenuated whole *P. falciparum* sporozoites. This idea is not novel as first experiments were conducted in the 1970s. Clyde and colleagues demonstrated that vaccination with radiation-attenuated sporozoites (RAS) could protect one out of three malaria-naïve volunteers from infection with *P. falciparum* through mosquito bites [83]. A milestone in the development of a whole sporozoite based vaccine was achieved when the US biotech company Sanaria Inc. managed to develop GMP-compliant methods to isolate, purify and cryopreserve *P. falciparum* sporozoites from infected mosquitoes. The direct inoculation by needle and syringe of their standardized manufactured product, named PfSPZ Vaccine, allows to move away from immunization via mosquito bite and to test the vaccine in the population most in need [84]. In order to elicit a strong immune response, most likely localized in the liver [85], PfSPZ Vaccine needs to be administered by direct venous inoculation (DVI) [86, 87]. While the clinical development of immunization with PfSPZ Vaccine is ongoing in the different age groups, alternative approaches of parasite attenuation are pursued. Immunization with fully infectious sporozoites under chloroquine coverage, an approach named PfSPZ-CVac, has shown promising results in malaria naïve volunteers [88]. Another approach uses genetically attenuated parasites (GAP), which leads to an arrest of parasite development in hepatocytes due to the deletion of three genes expressed during the pre-erythrocytic stage [89]. The current rationale of vaccinating with attenuated whole sporozoites is that the sporozoites will infect hepatocytes without being able to develop into merozoites that would induce asexual blood stage infection. Parasite attenuated by irradiation will stop early after hepatocyte infection since they cannot replicate their DNA strands. Parasites attenuated by chloroquine (prevention of blood stage infection) or genetically modified to knock-out essential genes, will develop within hepatocytes

until later stages. Therefore, the immune system might be exposed to a broader repertoire of antigens which could be beneficial for inducing effective and long-term immune responses using fewer sporozoites [67].

The identification of immunological mechanisms mediating protection is still ongoing. Immunization with PfSPZ Vaccine induces both, antibodies against PfCSP [90, 91] as well as liver-resident *P. falciparum* specific CD8 T-cells in monkeys [85, 90]. Liver-resident cells which are involved in inducing cellular-mediated immunity upon vaccination with PfSPZ Vaccine are difficult to access in peripheral blood in human subjects and therefore no clear correlate of protection has been found so far [67]. The humoral part of the immune response is better accessible. Serum from Tanzanian PfSPZ Vaccine-immunized volunteers was used to demonstrate that IgM antibodies can inhibit *P. falciparum* sporozoite invasion into hepatocytes *in vitro* and fix complement on sporozoites [92]. Furthermore, purified IgG from malaria-naïve PfSPZ vaccinated volunteers reduced liver infection significantly in humanized liver-chimeric mouse models [90].

While a high protective efficacy of PfSPZ Vaccine in malaria-naïve volunteers assessed by homologous CHMI was observed [93], clinical trials conducted in malaria pre-exposed populations resulted in reduced vaccine efficacy [94]. The outcomes of additional trials, evaluating PfSPZ Vaccine, conducted in Tanzania and Equatorial Guinea are discussed in Chapter 2. Currently, the planning for an already financed phase 3 clinical trial on Bioko Island, Equatorial Guinea is undergoing. The first of a total 2100 volunteers is planned to be immunized with PfSPZ Vaccine in the first quarter of 2020 [95].

## 1.3 Controlled Human Malaria Infections (CHMI)

### 1.3.1 CHMI: One hundred years of experience

Controlled Human Malaria Infection, the deliberate inoculation of infective sporozoites or merozoites into human volunteers, has been practiced since the early 20<sup>th</sup> century. Before the introduction of penicillin, the induction of fever caused by artificial infection with *Plasmodium* spp. parasites was the most effective treatment for neurosyphilis [96]. Only a few years after the first trials the so-called malariotherapy was fully accepted by the scientific community and in 1927 even awarded with the Nobel Prize [97]. Clinical and parasitological data obtained from the era of malariotherapy has provided invaluable insight into the human immune response and parasite growth dynamics, which remains informative a century later [98]. The first use of CHMI to assess vaccine efficacy was published in 1973 by David Clyde [83].

Today, variations of several CHMI models using different stages of the parasite are used to infect humans with the aim to evaluate the efficacy of different types of vaccines. Infection with sporozoites is mediated by bites of infected mosquitoes [99] or by injection of purified, cryopreserved live sporozoites [86]. Alternatively, blood-stage parasites can be used for CHMI by intravenous inoculation with infected erythrocytes [100].

For obvious reasons, most CHMI studies are focusing on *P. falciparum*, but non-*falciparum* malaria CHMI models are also being developed. Different clinical trial sites have successfully established *P. vivax* CHMI models [101]. Recently, it was demonstrated that the controlled infection of two volunteers with cryopreserved *P. malariae*-infected erythrocytes was well tolerated and led to stable parasitaemia [102]. No modern CHMI platform for infecting humans with *P. ovale* spp. and *P. knowlesi* has been established so far to our knowledge.

### 1.3.2 An accelerator for vaccine and drug development?

CHMI is increasingly being used to assess the efficacy of malaria vaccines and therefore contributes to acceleration in malaria vaccine development [103]. Based on the data from small CHMI trials an iterative process of vaccine and immunisation regimen optimization can be followed, which reduces the chance of late failures of candidates pushed into large and expensive phase 2 and 3 field vaccine trials [104]. Additionally, CHMI studies also offer a well-controlled environment to rapidly assess the efficacy of antimalarial drugs [105]. Using qPCR assays for asexual blood stage parasite quantification failing drugs can be identified immediately which increases patients safety [106].

There are also challenges and limitations when it comes to conducting CHMI studies that can only be performed in adults. Further standardization of CHMI protocols is needed to allow comparison across different studies and clinical trial sites which has improved by using the PfSPZ Challenge at a fixed dose of 3200 sporozoites inoculated intravenously. To cover the genetic diversity of *P. falciparum* in the field, it is important to have several strains from different malaria endemic regions which could be used for CHMI [66, 107]. Currently, for sporozoite-based CHMI studies only a limited number of *P. falciparum* strains are available. *P. falciparum* strains of West-African origin (PfNF54 [108] and PfNF166.C8 [109]), of Brazilian (Pf7G8 [110]) and Cambodian origin (PfNF135.C10 [111]) were tested in humans. Depending on the availability of cultured parasites more strains for CHMI might become available in future.

### 1.3.3 CHMI as a tool to study host and parasite immunobiology

The African continent carries the highest burden of malaria and its population is most in need for effective malaria control measures. Conducting CHMIs in malaria endemic population would allow earlier assessment of vaccine efficacy in target populations and provide insight into the immunological responses associated with NAI [112]. Although there are reports from Kenya, Nigeria and Liberia where controlled malaria infections were conducted between 1954 and 1962 [113], most CHMI studies have been performed so far in malaria-naïve populations [112]. In the past, the challenge to conduct CHMIs in Africa was attributed to the lack of high security facilities able to rear *P. falciparum*-infected *Anopheles* mosquitoes [114]. This obstacle had been overcome by PfSPZ Challenge [115]. In 2012, we showed for the first time that inoculation of malaria pre-exposed Tanzanians with PfSPZ Challenge is safe, well tolerated and infective [114]. These findings were later confirmed in studies conducted in Kenya [113] and Gabon [116, 117] where volunteers from malaria endemic regions were successfully infected with PfSPZ Challenge. Two additional, so far unpublished clinical trials used CHMI in Mali (clinicaltrials.gov: NCT02996695 and NCT02627456).

Given the logistical and financial expenditure of conducting CHMI studies, the use of this platform should be extended to more basic research questions beyond the evaluation of vaccine and drug candidates. The implementation of research projects on host and parasite immune-biology embedded within CHMI studies should be encouraged. Data obtained from CHMI is not only critical for the evaluation of vaccine candidates but also provides an opportunity to study immunological responses to infection in a controlled setting. Furthermore, data on parasite growth dynamics allows analysing parasite-host interaction in the most relevant populations. CHMIs also offer a convenient platform to evaluate novel diagnostic approaches for malaria as shown in Chapter 3 of this thesis.

#### 1.3.4 Monitoring of parasitaemia during CHMI

Post CHMI, volunteers are closely monitored for signs and symptoms of malaria. Parasitaemia in whole blood is rigorously monitored and volunteers are treated before they develop high parasitaemia levels and severe symptoms. Historically, the appearance of blood-stage parasitaemia was diagnosed using TBS microscopy while recently the more sensitive qPCR method is used as the diagnostic test in CHMI for the primary study endpoint. Several CHMI conducting research sites have discussed the use and in particular the advantages of qPCR-based monitoring of parasitaemia during CHMI studies in malaria-naïve [118–122] and pre-exposed individuals [113, 121, 123]. In summary, the implementation of qPCR improves safety while reducing the clinical burden and costs without compromising the evaluation of protective efficacy of pre-erythrocytic vaccine candidates in malaria-naïve volunteers [118] and might be the only solution to identify all infected volunteers in malaria pre-exposed populations with varying levels of NAI [124]. Special attention needs to be paid to performance evaluation, quality control and external quality assessment of any diagnostic tool used in diagnosis of malaria during clinical trials and in particular during CHMI studies [125].

## 1.4 Aims of this thesis

Malaria control interventions, such as distribution of ITNs and wide-spread IRS, have led to a significant decrease in prevalence and mortality from 2010 to 2015. Alarming, since the year 2016 no further progress in reducing global malaria cases and deaths could be achieved. This is most likely the result of reduced public funding for malaria control and the appearance of insecticide resistant vectors and drug resistant parasites. Novel malaria control approaches, such as highly effective vaccines and improved diagnostic tools are needed to prevent a resurgence of malaria as has been seen in the 1970s.

The overall goal of this PhD thesis is to contribute to the development, validation and implementation of novel molecular diagnostic tools in malaria endemic countries targeting all human infective malaria species.

In part one, we aimed to closely monitor asexual blood stage parasites at the individual level including the following three objectives:

- i) To establish a robust laboratory-based qPCR-platform for molecular monitoring of asexual blood stage parasitaemia in volunteers residing in Tanzania and Equatorial Guinea.
- ii) To evaluate the efficacy of attenuated whole sporozoite vaccines (PfSPZ Vaccine and PfSPZ-CVac) by homologous CHMI in Tanzanian and Equatorial Guinean adults.
- iii) To study the impact of malaria pre-exposure on multiplication rates of *P. falciparum* parasites during CHMI.

In part two, we aimed to adapt and extend molecular diagnostic tools for improvement of malaria diagnosis for surveillance at the population level. The following objectives were defined:

- i) To develop novel high-throughput, field-deployable and highly sensitive tools and approaches for molecular malaria surveillance.
- ii) To include qPCR-based detection of *P. malariae* and *P. ovale* spp. parasites in active malaria surveillance in Tanzania and Equatorial Guinea.



# Chapter II

Molecular monitoring of asexual blood stage parasites  
at the individual level

## **2.1 PfSPZ Vaccine safety, immunogenicity and efficacy against CHMI in Tanzanian and Equato-Guinean volunteers**

A short review of all six clinical trials, which were the backbone of this PhD thesis is following below. The outcomes of the BSPZV3, EGSPZV2 and EGSPZV3 trials are not published yet.

### **BSPZV1 – Safety, immunogenicity and protective efficacy of PfSPZ Vaccine in Tanzanian adults**

The BSPZV1 study [124], with the identical vaccination schedule as studies in the USA [126] and Mali [94], showed that two different doses of PfSPZ Vaccine, five immunizations with  $1.35 \times 10^5$  or  $2.7 \times 10^5$  RAS, were safe and well tolerated in Tanzanian men. A dose-effect was observed. In the group receiving the lower dose, VE against homologous CHMI was 5.6% (1/18 volunteers protected), while in the higher dose group the VE was 20% (4/20 volunteers were protected). The five immunizations with  $2.7 \times 10^5$  RAS were less protective in Tanzanians than in Americans who received the same dosage and regimen of PfSPZ Vaccine [126]. VE was similar as seen in intense field exposure to heterogeneous *P. falciparum* parasites in Mali [94], suggesting that CHMI is a valuable tool to assess and predict vaccine efficacy in malaria pre-exposed populations. Antibody responses to PfCSP in Tanzanians were lower than in malaria-naïve Americans, but significantly higher than in Malians. Furthermore, all four volunteers from the high dose group which were protected against CHMI 3 weeks after the last vaccination, were still protected against a second CHMI 24 weeks post vaccination. Notably, the prepatent period assessed by qPCR of unprotected vaccinated vol-

unteers was significantly longer than the prepatent period in placebo controls, indicating a vaccine-induced reduction of sporozoites in the liver.

### **BSPZV2 – Safety and immunogenicity of PfSPZ Vaccine in Tanzanian adults, adolescents, children, and infants**

A total of 93 volunteer were enrolled into the BSPZV2 study [127]. Using an age-de-escalation study design, vaccinations started in adults, followed by adolescents, children and finally infants. PfSPZ Vaccine was safe and well tolerated in all age groups, including the infants. Immunogenicity was assessed in all age groups receiving three doses of  $9.0 \times 10^5$  RAS. Humoral immune responses against PfCSP were highest in infants and lowest in adults. T-cell responses were higher in children than in adults and completely absent in infants, suggesting that PfSPZ Vaccine may be more protective in children than in adults and infants.

Vaccine efficacy against CHMI was only assessed in adults. Two different doses, three immunizations with  $9.0 \times 10^5$  and  $1.8 \times 10^6$  RAS were tested against CHMI. Unexpected, higher VE (100%, 5/5 protected) was observed in the lower dose group compared to the group which received higher dose (33%, 2/6 protected). Humoral and cellular immune responses were higher in the high dose group which was less protective against CHMI. After a second CHMI, 37-40 weeks after last vaccination, only one volunteer from the high dose group remained protected.

### **BSPZV3 – Safety, immunogenicity and efficacy of PfSPZ Vaccine in HIV+ Tanzanian adults**

There is a significant geographic overlap between HIV and malaria in Sub-Saharan Africa [128]. Therefore, safety of malaria vaccine needs to be assessed in people living with HIV. We evaluated the safety, immunogenicity and protective efficacy in HIV+ Tanzanian volunteers. Four doses of  $9.0 \times 10^5$  RAS within one week followed by a booster vaccination at day 28 were tested in two groups, consisting of 12 HIV+ and 9 HIV- volunteers. The HIV+ volunteers were enrolled based on documented HIV infection at WHO clinical stage 1. They were all on stable anti-retroviral therapy for at least 3 months and had CD4 counts above 500 cells/ $\mu$ L at screening. CD4 counts and HIV-1 viral loads were monitored closely throughout the entire trial. The outcomes of this trials are not published yet but based on a pre-liminary analysis of the data it can be concluded that the PfSPZ Vaccine was safe and well tolerated in HIV+ individuals. Antibody responses to PfCSP were significantly lower in HIV+ volunteers (S. L. Hofmann, personal communication). None of the HIV+ volunteers were protected upon CHMI, while the VE in the HIV- group was 83.3% (5/6). Although, the HIV+ volunteers were on anti-retroviral therapy with stable CD4 counts and HIV-1 viral loads, this result suggests that their immune system is still compromised to a certain degree which impairs immune responses induced by PfSPZ Vaccine.

## **EGSPZV1 - Safety and immunogenicity of PfSPZ Vaccine in Equatorial Guineas first clinical trial**

In the first clinical trial ever conducted in Equatorial Guinea, the safety and immunogenicity of three doses of  $2.7 \times 10^5$  RAS was tested in 20 adult male volunteers [129]. The vaccine was well tolerated, but antibody responses to PfCSP were significantly lower than responses in US or Tanzanian adults who received the same dosage regimen. Although, it was a small trial its importance for Equatorial Guinea's malaria research efforts should not be underestimated. This trial is the foundation for development of a robust research and development program to eliminate malaria on Bioko Island using a malaria vaccine as a complimentary tool to already ongoing malaria control efforts.

## **EGSPZV2 - Safety and immunogenicity of PfSPZ Vaccine in Equato-Guinean adults, adolescents, children, and infants and comparison between PfSPZ Vaccine and PfSPZ-CVac for efficacy against CHMI**

Compared to the BSPZV2 study, higher doses of PfSPZ Vaccine were tested. Adults received three doses of  $2.7 \times 10^6$  RAS, while younger children (1-5 years old) and infants received three doses of  $1.8 \times 10^6$  RAS. These increased PfSPZ Vaccine doses were safe and well tolerated in all age groups. The approach of using three doses of 100'000 non-attenuated sporozoites administered under chloroquine prophylaxis, resulted in transient parasitaemia (ranging 100-1000 parasites/mL) in whole blood 7 – 9 days post vaccination similar to what had been observed in malaria naïve German volunteers [88]. The direct, side-by-side comparison of vaccination with RAS and PfSPZ-CVac in Equato-Guinean volunteers revealed higher protective efficacy among volunteers who received PfSPZ-CVac, with VE of 33% (6/18) for

RAS versus 57% (8/14) for PfSPZ-CVac. The analysis of immunological correlates of protection is still ongoing.

### **EGSPZV3 – Optimization of PfSPZ Vaccine dosage regimen in Equato-Guinean adults**

The EGSPZV3 study was designed to compare the administration of four different regimens of PfSPZ Vaccine for vaccine efficacy against homologous CHMI eight weeks after last vaccination. The focus was to establish which of the condensed regimens, completing all required immunizations within four weeks or less is optimal to be used during the upcoming phase 3 clinical trial planned for 2020 on Bioko Island. This trial is not unblinded yet, therefore no conclusion about the different vaccination schedules can be made at this time. However, indications are that the vaccination dose of  $9 \times 10^5$  RAS applied at days 1, 8 and 28 is superior to the other regimen tested.

This chapter contains the following publications:

**Jongo *et al.* Safety, immunogenicity, and protective efficacy against controlled human malaria infection of plasmodium falciparum sporozoite vaccine in Tanzanian adults.**

*The American Journal of Tropical Medicine and Hygiene* 2018

**Olotu *et al.* Advancing Global Health through Development and Clinical Trials Partnerships: A Randomized, Placebo-Controlled, Double-Blind Assessment of Safety, Tolerability, and Immunogenicity of PfSPZ Vaccine for Malaria in Healthy Equatoguinean Men.** *The American Journal of Tropical Medicine and Hygiene* 2018

**Jongo *et al.* Safety and Differential Antibody and T-Cell Responses to Plasmodium falciparum Sporozoite Vaccine by Age in Tanzanian Adults, Adolescents, Children, and Infants.** *The American Journal of Tropical Medicine and Hygiene* 2019

**Schindler *et al.* Controlled Human Malaria Infections in Tanzania and Equatorial Guinea: The impact of malaria pre-exposure on diagnosis and multiplication rate of *P. falciparum* parasites.** *manuscript in preparation* 2019

## Safety, Immunogenicity, and Protective Efficacy against Controlled Human Malaria Infection of *Plasmodium falciparum* Sporozoite Vaccine in Tanzanian Adults

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**Abstract.** We are using controlled human malaria infection (CHMI) by direct venous inoculation (DVI) of cryopreserved, infectious *Plasmodium falciparum* (Pf) sporozoites (SPZ) (PfSPZ Challenge) to try to reduce time and costs of developing PfSPZ Vaccine to prevent malaria in Africa. Immunization with five doses at 0, 4, 8, 12, and 20 weeks of  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine gave 65% vaccine efficacy (VE) at 24 weeks against mosquito bite CHMI in U.S. adults and 52% (time to event) or 29% (proportional) VE over 24 weeks against naturally transmitted Pf in Malian adults. We assessed the identical regimen in Tanzanians for VE against PfSPZ Challenge. Twenty- to thirty-year-old men were randomized to receive five doses normal saline or PfSPZ Vaccine in a double-blind trial. Vaccine efficacy was assessed 3 and 24 weeks later. Adverse events were similar in vaccinees and controls. Antibody responses to Pf circumsporozoite protein were significantly lower than in malaria-naïve Americans, but significantly higher than in Malians. All 18 controls developed Pf parasitemia after CHMI. Four of 20 (20%) vaccinees remained uninfected after 3 week CHMI ( $P = 0.015$  by time to event,  $P = 0.543$  by proportional analysis) and all four (100%) were uninfected after repeat 24 week CHMI ( $P = 0.005$  by proportional,  $P = 0.004$  by time to event analysis). *Plasmodium falciparum* SPZ Vaccine was safe, well tolerated, and induced durable VE in four subjects. Controlled human malaria infection by DVI of PfSPZ Challenge appeared more stringent over 24 weeks than mosquito bite CHMI in United States or natural exposure in Malian adults, thereby providing a rigorous test of VE in Africa.

### INTRODUCTION

In 2015 and in 2016, there were an estimated 429,000–730,500 deaths caused by malaria.<sup>1–3</sup> *Plasmodium falciparum* (Pf) is the cause of > 98% of malaria deaths and > 80% of malaria cases in sub-Saharan Africa. Our goal is to field a vaccine that will prevent infection with Pf and thereby prevent all manifestations of Pf malaria and parasite transmission from humans to mosquitoes.<sup>4</sup>

*Plasmodium falciparum* sporozoites (SPZ) are the only immunogens that have ever prevented Pf infection in > 90% of subjects.<sup>5–7</sup> Sanaria® PfSPZ Vaccine (Sanaria Inc., Rockville, MD) is composed of radiation-attenuated, aseptic, purified, cryopreserved PfSPZ.<sup>8,9</sup> When administered by rapid intravenous injection, PfSPZ Vaccine protected 100% (6/6) of malaria-naïve subjects in the United States against mosquito bite-controlled human malaria infection (CHMI) with Pf parasites similar to those in the vaccine (homologous) 3 weeks after the last immunization,<sup>10</sup> and 65% at 24 weeks.<sup>11</sup> Protection was durable against homologous mosquito bite CHMI for at least 59 weeks<sup>12</sup> and heterologous (parasites different than in vaccine) mosquito bite CHMI for at least 33 weeks.<sup>13</sup> PfSPZ Vaccine also prevented naturally transmitted heterogeneous Pf in adults in Mali for at least 24 weeks (vaccine efficacy [VE] 52% by time to event and 29% by proportional analysis).<sup>14</sup>

We used the same dosage regimen as in the United States and Mali to evaluate the tolerability, safety, immunogenicity, and VE of PfSPZ Vaccine in young adult male Tanzanians.

Previously, we had conducted the first modern CHMI in Africa and showed that injection of aseptic, purified, cryopreserved PfSPZ, Sanaria® PfSPZ Challenge, consistently infected Tanzanian volunteers and subsequently repeated in multiple other countries.<sup>15–21</sup> In this study, we took advantage of this capability to assess VE of PfSPZ Vaccine by CHMI with PfSPZ Challenge (NF54). The same PfSPZ Vaccine dosage regimen was less immunogenic and protective in Tanzanians than in Americans,<sup>11</sup> and VE against homologous CHMI in Tanzania was lower (or similar) to VE against intense field exposure to heterogeneous Pf parasites in Mali.<sup>14</sup>

### MATERIAL AND METHODS

**Study design and population.** This double-blind, randomized, controlled trial was conducted in Bagamoyo, Tanzania, between April 2014 and August 2015. Sixty-seven healthy male volunteers of 18–35 years of age were recruited from higher learning institutions in Dar es Salaam. After initial screening, prospective volunteers were invited to the Bagamoyo Clinical Trial Unit of the Ifakara Health Institute (IHI) to complete informed consent and screening.

All had to complete a 20-question assessment of trial understanding with a 100% correct response rate on the first or second attempt (Supplemental Table 1) to be eligible. Volunteers were screened using predetermined inclusion and exclusion criteria (Supplemental Tables 2 and 3). History of malaria in the previous 5 years or antibodies to Pf exported protein 1 (PfEXP1) by an enzyme-linked immunosorbent assay (ELISA) above a level associated with a single, recent Pf infection by CHMI<sup>19</sup> (see the Antibody assays section) were the exclusion criteria. Hematology, biochemistry, and parasitology testing, including malaria thick blood smear (TBS), stool,

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and urine by microscopy was carried out. Tests for human immunodeficiency virus and hepatitis B and C were performed after counseling; volunteers were excluded if positive and referred for evaluation and management by appropriate local physicians. Volunteers were excluded if they had significant abnormalities on electrocardiograms.

The trial was performed in accordance with Good Clinical Practices. The protocol was approved by institutional review boards (IRBs) of the IHI (Ref. No. IHI/IRB/No:02-2014), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/1691), the Ethikkommission Nordwest-und Zentralschweiz, Basel, Switzerland (reference number 261/13), and by the Tanzania Food and Drug Authority (Ref. No. TFDA 13/CTR/0003); registered at Clinical Trials.gov (NCT02132299); and conducted under U.S. FDA IND application.

**Investigational products (IPs).** The IPs were Sanaria® PfSPZ Vaccine<sup>8–14</sup> and Sanaria® PfSPZ Challenge.<sup>15–20</sup> PfSPZ Vaccine consists of aseptic, purified, vialled, metabolically active, nonreplicating (radiation attenuated), cryopreserved PfSPZ (NF54 strain). It was stored, thawed, diluted, and administered by direct venous inoculation (DVI) in 0.5 mL through a 25-gauge needle.<sup>11,14,18,20</sup> PfSPZ Challenge is identical to PfSPZ Vaccine except it is not radiation attenuated. It was handled and administered like PfSPZ Vaccine. Preparation of IPs was supervised by the study pharmacist. After labeling the syringe, the pharmacist handed it to the clinical team through a window.

**Allocation and randomization.** Volunteers were allocated to five groups (Table 1; Figure 1). Forty-nine received PfSPZ Vaccine and eight normal saline (NS). Ten were additional infectivity controls. The clinical team and volunteers were blinded to assignment to vaccine or NS until study end.

**Group 1.** Three volunteers received consecutive doses of  $3 \times 10^4$ ,  $1.35 \times 10^5$ , and  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine at 4-week intervals to assess safety (Group 1).

**Groups 2 and 3.** Volunteers were randomized to receive  $1.35 \times 10^5$  PfSPZ of PfSPZ Vaccine ( $N = 20$ ) or NS ( $N = 4$ ) (Group 2), or  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine ( $N = 20$ ) or NS ( $N = 4$ ) (Group 3) at 0, 4, 8, 12, and 20 weeks.

**Group 4.** Six volunteers were immunized with  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine on the same schedule as Group 3.

**Group 5.** Ten volunteers served as unblinded infectivity controls during CHMIs (see in the following paragraph): two with CHMI #1, two with CHMI #2, and six with CHMI #3.

**Vaccine efficacy.** *Controlled human malaria infection.* Vaccine efficacy was assessed by CHMI by DVI of  $3.2 \times 10^3$  PfSPZ of PfSPZ Challenge. Controlled human malaria infection #1 was 3 weeks after the last immunization in Group 2. Controlled human malaria infection #2 was 3 weeks after the

last immunization in Group 3. Controlled human malaria infection #3 was 24 weeks after the last immunization in Groups 3 and 4 and included the four volunteers in Group 3 who did not develop parasitemia after CHMI #2 and the six Group 4 volunteers. Volunteers were inpatients from day 9 after PfSPZ Challenge injection for observation until diagnosed and treated for malaria or until day 21; daily outpatient monitoring for TBS-negative volunteers continued until day 28. Thick blood smears were obtained every 12 hours on days 9–14 after CHMI and daily on days 15–21 until positive or until day 21. Thick blood smears could be performed more frequently, if volunteers had symptoms/signs consistent with malaria. After initiation of treatment, TBSs were assessed until two consecutive daily TBSs were negative and on day 28.

**Detection of *Pf* parasites and parasite DNA.** Slide preparation and reading for TBSs were performed as described.<sup>19</sup> Sensitivity was 2 parasites/ $\mu$ L blood unless the volunteer was symptomatic, in which case four times as many fields were read. Parasitemia was also determined by quantitative polymerase chain reaction (qPCR) with sensitivity of 0.1 parasites/ $\mu$ L blood based on a multiplex assay detecting *Plasmodium* spp. 18S genes and the human RNaseP gene as endogenous control.<sup>22</sup> A second, more sensitive qPCR assay with a sensitivity of 0.05 parasites/ $\mu$ L blood and targeting the Pf-specific telomere-associated repetitive element 2<sup>23</sup> was used to reanalyze all samples that were negative by 18S-based qPCR. After the start of CHMI, the time of first blood sample positivity by qPCR was used to determine infection status and for the calculation of prepatent period. Volunteers were continuously monitored by qPCR until malaria treatment based on TBS positivity. The World Health Organization International Standard for Pf DNA Nucleic Acid Amplification Techniques (NIBSC, Hertfordshire, United Kingdom) was used as standard for calculation of parasite densities. DNA was extracted from 100  $\mu$ L whole blood and eluted with 50  $\mu$ L Elution Buffer using Quick-gDNA Blood MicroPrep Kit (Zymo Research, Irvine, CA). Blood samples were analyzed retrospectively by qPCR after storing at  $-80^\circ\text{C}$  after the conclusion of CHMIs. To exclude field strain infections, parasite genotyping was performed on samples randomly chosen as described.<sup>24</sup> In all cases in which TBS was negative and qPCR was considered positive, two consecutive samples were positive by qPCR.

**Adverse events (AEs).** Volunteers were observed as inpatients for 48 hours after administration of IP and discharged with diaries and thermometers for recording AEs and temperatures and followed with daily telephone calls. Symptoms and signs (solicited and unsolicited) were recorded and graded by physicians: mild (easily tolerated), moderate (interfere with normal activity), severe (prevents normal activity),

TABLE 1  
Demographic characteristics of volunteers

	Vaccinees	Normal saline controls	Infectivity controls
Number of volunteers	49	8	10
Percentage males	100%	100%	100%
Mean age in years (range)	24 (20, 30)	23 (20, 28)	25 (21, 28)
Percentage Africans	100%	100%	100%
Mean body mass index (range)	22.33 (18.00, 29.70)	21.91 (19.00, 24.20)	21.68 (18.40, 24.30)
Number (%) heterozygous for alpha thalassemia	22 (44.9%)	4 (50%)	5 (50%)
Number (%) with LTBI* (QuantIFERON positive)	17 (34.7%)	3 (36.5%)	1 (10%)
Number (%) positive on screening of urine or stool for parasitic infection	0 (0%)	1 (12.5%)	0 (0%)
Number (%) students	49 (100%)	8 (100%)	10 (100%)

\* Latent tuberculosis infection.

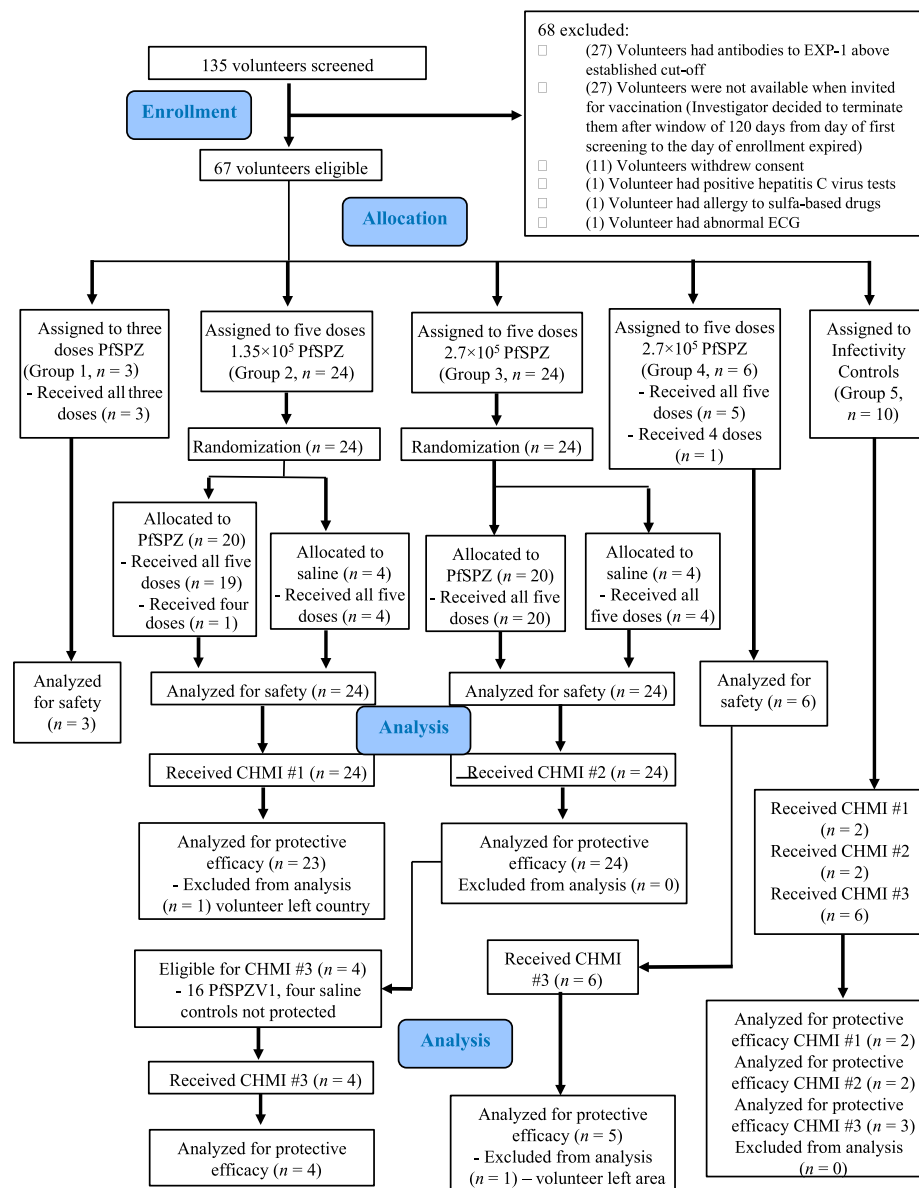


FIGURE 1. Volunteer participation (CONSORT 2010 diagram). This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

or life threatening. Axillary temperature was grade 1 (> 37.5–38.0°C), grade 2 (> 38.0–39.0°C), grade 3 (> 39.0–40.0°C), or grade 4 (> 40.0°C). Hematological and biochemical abnormalities were also assessed using standard clinical assays.

During the first 7 days after injection of IPs, prespecified local (site of injection) and systemic AEs were solicited. Open-ended questioning was used to identify unsolicited AEs through day 28 (Supplemental Table 4). All AEs were assessed for severity and relatedness to IP administration. Adverse events were classified as definitely related, probably related, possibly related, unlikely to be related, and not related. Definitely, probably, and possibly were considered to be related. Unlikely to be related and not related were considered to be unrelated. For CHMIs, volunteers returned on day 9 for admission to the ward for diagnosis and treatment of malaria. Events during the 8–28 day period were assessed for relationship to Pf infection and considered related if the event was within 3 days before and 7 days after TBS was first positive.

**Antibody assays.** Sera were assessed for antibodies by ELISA, immunofluorescence assay (IFA), and inhibition of sporozoite invasion (ISI) assay as described (see Supplemental Table 5).<sup>25</sup> For ELISAs, the results are reported as the serum dilution at which the optical density (OD) was 1.0. Enzyme-linked immunosorbent assay for PfEXP1 was used to screen volunteers for possible malaria exposure (Supplemental Table 6). Any subject with an OD 1.0 of  $\geq 600$  was excluded. This was because we had previously determined in Tanzanians who underwent CHMI<sup>19</sup> that antibodies to PfEXP1 at this level were a sensitive indicator of recent Pf infection (unpublished).

**T-cell assays.** T-cell responses in cryopreserved peripheral blood mononuclear cells (PBMC) were measured by flow cytometry in a single batch after the study as described.<sup>12</sup> After stimulation, cells were stained as described.<sup>26</sup> The staining panels are in Supplemental Table 7 and antibody clones and manufacturers are in Supplemental Table 8. All antigen-specific frequencies are reported after background

subtraction of identical gates from the same sample incubated with control antigen. Data were analyzed with FlowJo v9.9.3 (TreeStar, Ashland, OR) and graphed in Prism v7.0a (Graph-Pad, San Diego, CA).

**Statistical analysis.** Comparisons of categorical variables between groups were analyzed using 2-tailed Fisher's exact test. Comparisons of continuous variables between groups were analyzed by 2-tailed nonparametric tests. For multiple group comparisons, the Kruskal–Wallis test was used. Time to event was assessed by the Kaplan–Meier curves and log-rank test. Vaccine efficacy by time to event was quantified using Cox proportional hazards ratios. Time to event data were analyzed from CHMI injection until positive TBS result or positive qPCR result. Controlled human malaria infection follow-up period lasted until day 28 after CHMI injection. Analyses of immunological data are described with the data.

**Role of the funding source.** The funders were involved in study design, study management, data collection, data analysis, data interpretation, and writing the report. Salim Abdulla and Stephen L. Hoffman had full access to all data in the study and final responsibility for decision to submit for publication.

## RESULTS

**Study population and experience with DVI.** Fifty-seven Tanzanian men (Table 1; Figure 1) met the criteria (Supplemental Tables 2 and 3) and received PfSPZ Vaccine ( $N = 49$ ) or NS ( $N = 8$ ). All volunteers had AA hemoglobin and normal G6PD activity. Thirty-one volunteers (46%) were heterozygous for  $\alpha$ -thalassemia; 21 had evidence of latent tuberculosis infection by Quantiferon testing, but showed no evidence of active tuberculosis. One volunteer (group 2, NS) had *Strongyloides stercoralis* on screening and was successfully treated before vaccination (Table 1).

Of 237 immunizations with PfSPZ Vaccine, 234 were completed with a single injection (98.7%). Two hundred and thirty

injections (97.0%) were considered painless by the volunteer. For NS subjects, 39 of 40 immunizations (97.5%) were completed in a single injection and 39 of 40 (97.5%) considered painless by the volunteer. The nurse performing immunizations considered the procedure to be simple in 265 of 273 single injections (97.1%).

One subject in Group 2 received four immunizations. The third immunization was withheld while the subject was evaluated for what was diagnosed as benign ethnic neutropenia.<sup>27,28</sup> One subject in Group 4 missed his second immunization when he left town. All other subjects (other than Group 1 and added infectivity controls) received five immunizations.

**Safety.** Among 49 volunteers who received 237 doses of PfSPZ Vaccine, there were 17 solicited AEs possibly related to IP (17/237 = 7.2%) in 10 of the 49 vaccinees (20.4%) (Table 2). Among eight volunteers who received 40 doses of NS, there were two solicited AEs possibly related to IP (2/40 = 5.0%) in one of the eight controls (12.5%) (Table 2). There were no AEs considered by the clinicians to be probably or definitely related to IP. There were no local or serious AEs. One episode each of headache and fever were grade 2; all other solicited AEs were grade 1. None of the comparisons of AEs between vaccinees and controls or between Group 2 ( $1.35 \times 10^5$  PfSPZ) and Groups 3 and 4 ( $2.7 \times 10^5$  PfSPZ) showed statistically significant differences (Table 2). Twenty-six of 49 vaccinees (53.1%) experienced 43 unsolicited AEs (0.88/individual) in the 28 days following injections #1–#4 and the 21 days before CHMI after injection #5. Seven of eight controls (87.5%) experienced 14 unsolicited AEs (2/individual) during this period. None of these unsolicited AEs recorded within 28 days of an immunization was considered related to IP.

Laboratory abnormalities occurred at roughly equal rates comparing PfSPZ Vaccine recipients and controls, except for leukocytosis and eosinophilia, which were more frequent in controls (Table 3). There was no apparent explanation for these differences. A cyclic variation in total bilirubin following

TABLE 2  
Solicited AEs by group considered possibly\* related to administration of the investigational product during the first 7 days post immunization

	Group 1 (dose escalation)	Group 2 ( $1.35 \times 10^5$ PfSPZ)	Group 3 ( $2.7 \times 10^5$ PfSPZ)	Group 4 ( $2.7 \times 10^5$ PfSPZ)	Total PfSPZ vaccine	NS controls
Number of volunteers	3	20	20	6	49	8
Total number of injections	9	99	100	29	237	40
Number of local AEs	0	0	0	0	0	0
Numbers of systemic AEs (% of total immunizations)						
All	1 (11%)	10 (10.1%)	6 (6%)	0	17 (7.2%)	2 (5.0%)
Headache*	1 (11%)	7 (7%)†	2 (2%)	0	10 (4.2%)	1 (2.5%)
Abdominal pain	0	2 (2%)	1 (1%)	0	3 (1.3%)	0
Chills	0	0	1 (1%)	0	1 (0.4%)	0
Fever	0	0	2 (2%)	0	2 (0.8%)	0
Diarrhea	0	0	0	0	0	1 (2.5%)
Chest pain	0	1 (1%)	0	0	1 (0.4%)	0
Other	0	0	0	0	0	0
Systemic AEs - no. volunteers with $\geq 1$ event (% of volunteers)						
Any	1 (33%)	7 (35%)	2 (10%)	0	10 (20.4%)	1 (13%)
Headache	1 (33%)	6 (30%)	2 (10%)	0	9 (18.4%)	1 (13%)
Abdominal pain	0	2 (10%)	1 (5%)	0	3 (6.1%)	0
Chills	0	0	1 (5%)	0	1 (2.0%)	0
Fever	0	0	2 (10%)	0	2 (4.1%)	0
Diarrhea	0	0	0	0	0	1 (13%)
Chest pain	0	1 (5%)	0	0	1 (2.0%)	0
All other	0	0	0	0	0	0

AEs = adverse events; PfSPZ = *Plasmodium falciparum* sporozoites. There were no significant differences between vaccinees as compared with normal saline (NS) controls for any or all AEs. All AEs were grade 1, except one headache and one fever. Local solicited AEs: injection site pain, tenderness, erythema, swelling, or induration. Systemic solicited AEs: allergic reaction (rash, pruritus, wheezing, shortness of breath, bronchospasm, allergy-related edema/angioedema, hypotension, and anaphylaxis), abdominal pain, arthralgia, chest pain/discomfort, chills, diarrhea, fatigue, fever, headache, malaise, myalgia, nausea, pain (other), palpitations, shortness of breath, and vomiting.

\* All AEs were considered possibly related. None were considered probably or definitely related.

† 4/7 episodes of headache occurred after the third vaccine dose and did not recur with fourth or fifth doses. No factor was identified to account for this apparent clustering of headache.

each immunization was observed equally in volunteers receiving vaccine or NS that was attributed to enriched diet, as the volunteers were transported to Bagamoyo from Dar es Salaam during the periods of immunization and CHMI and were amply fed (see Supplemental Figure 1). In Dar es Salaam, malaria transmission is low. No volunteer had malaria during screening or during the trial other than from CHMI.

**Tolerability, safety, and VE during CHMI.** Forty-six vaccinees, eight NS controls, and 10 added infectivity controls underwent homologous CHMI. All subjects were negative by TBS and qPCR for Pf infection on the day of CHMI. Two volunteers were excluded from primary analysis—a Group 2 volunteer who left the area 2 days after administration of PfSPZ Challenge and a Group 4 volunteer who left 9 days after. Both volunteers were located and treated preemptively.

**Tolerability and safety of administration of PfSPZ challenge.** Controlled human malaria infection was well tolerated with no local solicited AEs and three systemic solicited AEs (grade 1 headache in Group 3, grade 2 headache in Group 4, and grade 1 arthralgia in an infectivity control) in the 7 days post-administration of PfSPZ Challenge.

**Parasitemia.** Controls. The 18 NS and infectivity controls developed Pf infection after CHMI (16 TBS and qPCR positive and two TBS negative and qPCR positive) (Figure 2A–D and Supplemental Table 9). These included four NS and two infectivity controls in CHMI #1, the same in CHMI #2, and six infectivity controls in CHMI #3. All received the same lot of PfSPZ Challenge. One isolate of those positive from CHMI #1, one from CHMI #2, and four from CHMI #3 were genotyped,<sup>24</sup> and all parasites tested were PfNF54. Vaccine efficacy was calculated based on the results of qPCR assays from the six controls in CHMI #1, CHMI #2, and CHMI #3 individually (Figure 2D).

Group 2 ( $1.35 \times 10^5$  PfSPZ). Seventeen of 18 volunteers who received five doses and 1/1 volunteer who received four doses developed parasitemia (Figure 2A), 15 positive by TBS and qPCR, and 3 by qPCR only (CHMI #1) (Supplemental Table 10). One volunteer was negative through day 28 by TBS and qPCR. Vaccine efficacy by proportional analysis

was 5.56% (95% confidence interval [CI]: 3.61%, 14.73%;  $P > 0.99$ , Fisher's exact test, 2-tailed). There was no significant delay in parasitemia by qPCR in the vaccinees as compared with controls ( $P = 0.4481$  by log rank).

Group 3 ( $2.7 \times 10^5$  PfSPZ). First CHMI at 3 weeks (CHMI #2): 16/20 volunteers who received five doses developed parasitemia (Figure 2B), all positive by TBS and qPCR; four volunteers were negative through day 28 by TBS and qPCR. Vaccine efficacy by proportional analysis was 20% (95% CI: 4.62%, 35.38%;  $P = 0.543$ ). There was a delay in the onset of parasitemia in vaccinees as compared with controls ( $P = 0.015$  by log rank).

Second CHMI at 24 weeks (CHMI #3): The four uninfected volunteers from the first CHMI underwent a second CHMI 24 weeks after the last vaccine dose (Figure 2C). Three were negative by TBS and qPCR through day 28 day. The fourth volunteer, who was asymptomatic, was reported to have a positive TBS on day 12 and treated. The sample with positive TBS was negative by retrospective qPCR. Reevaluation of the TBS indicated an error in slide reading (false-positive). Vaccine efficacy by proportional analysis at this time point was 100% (for 3/3 and 4/4 protected: 95% CI: 43.8%, 100%, and 51.01%, 100%;  $P = 0.012$  and 0.005, respectively). However, given the 20% VE at 3 weeks by proportional analysis, overall VE by proportional analysis was considered to be 20%.

Group 4 ( $2.7 \times 10^5$  PfSPZ). First CHMI at 24 weeks after the last vaccine dose (CHMI #3): 4/5 vaccinees developed parasitemia by TBS and qPCR. The fifth was negative by TBS, but positive by qPCR (see Supplemental Table 10). There was one excluded volunteer (see the previous paragraph). Vaccine efficacy by proportional analysis was 0% ( $P > 0.99\%$ ). There was a significant delay in the onset of parasitemia by qPCR in vaccinees as compared with controls ( $P = 0.001$  by log rank).

**$\alpha$ -thalassemia.** Volunteers heterozygous for  $\alpha$ -thalassemia were no more likely to be TBS negative and qPCR positive than volunteers without  $\alpha$ -thalassemia (three of 27 versus three of 34,  $P = 1.0$ ). Protection from CHMI did not correlate with  $\alpha$ -thalassemia status; 3/37 with normal hemoglobin and 2/29 heterozygous for  $\alpha$ -thalassemia were protected.

TABLE 3  
Summary of abnormal laboratory values and severity grades

Laboratory parameter	Vaccinees in Group 2 ( $1.35 \times 10^5$ PfSPZ) (N = 20)		Vaccinees in groups 3 and 4 ( $2.7 \times 10^5$ PfSPZ) (N = 26)		NS controls (N = 8)		P values: vaccinees (N = 46) vs. controls (N = 8)
	No.	%	No.	%	No.	%	
Leukocytosis	1	5	2	7.7	3	37.5	0.0358
Leukopenia	6	30	7	27	1	12.5	> 0.05
Neutropenia	6	30	5	19	2	25	> 0.05
Lymphopenia	3	15	3	11.5	2	25	> 0.05
Eosinophilia	0	0	2	7.7	3	37.5	0.0194
Decreased hemoglobin	1	5	0	0	0	0	> 0.05
Thrombocytopenia	1	5	0	0	0	0	> 0.05
Elevated creatinine	2	10	4	15.4	2	25	> 0.05
Low total bilirubin	4	20	7	27	1	12.5	> 0.05
Elevated total bilirubin	2	10	2	7.7	2	25	> 0.05
Elevated alkaline phosphatase	1	5	2	7.7	0	0	> 0.05
Elevated alanine aminotransferase	3	15	5	19	2	25	> 0.05
Elevated aspartate aminotransferase	0	0	3	11.5	0	0	> 0.05

PfSPZ = *Plasmodium falciparum* sporozoites. P values calculated using Fisher's exact test (2-tailed). One volunteer who received saline developed Grade 3 eosinophilia attributed to *Strongyloides stercoralis* infection, which improved with anthelmintic therapy. This volunteer had a baseline of mild eosinophilia, which persisted throughout the clinical trial. All other laboratory abnormalities were Grade 2 or less. There was no association between laboratory abnormalities and time after a dose or increasing number of doses. Three abnormalities during immunization were deemed clinically significant or Grade 3. One was diagnosed as benign ethnic neutropenia, one was lymphopenia associated with an infected foot laceration, and one was eosinophilia associated with *Fasciolopsis buski* and *S. stercoralis* infection. Lymphopenia and eosinophilia resolved with treatment. Two Group 4 volunteers had asymptomatic hookworm infections diagnosed before controlled human malaria infection; one was coinfecting with *Enterobius vermicularis*.

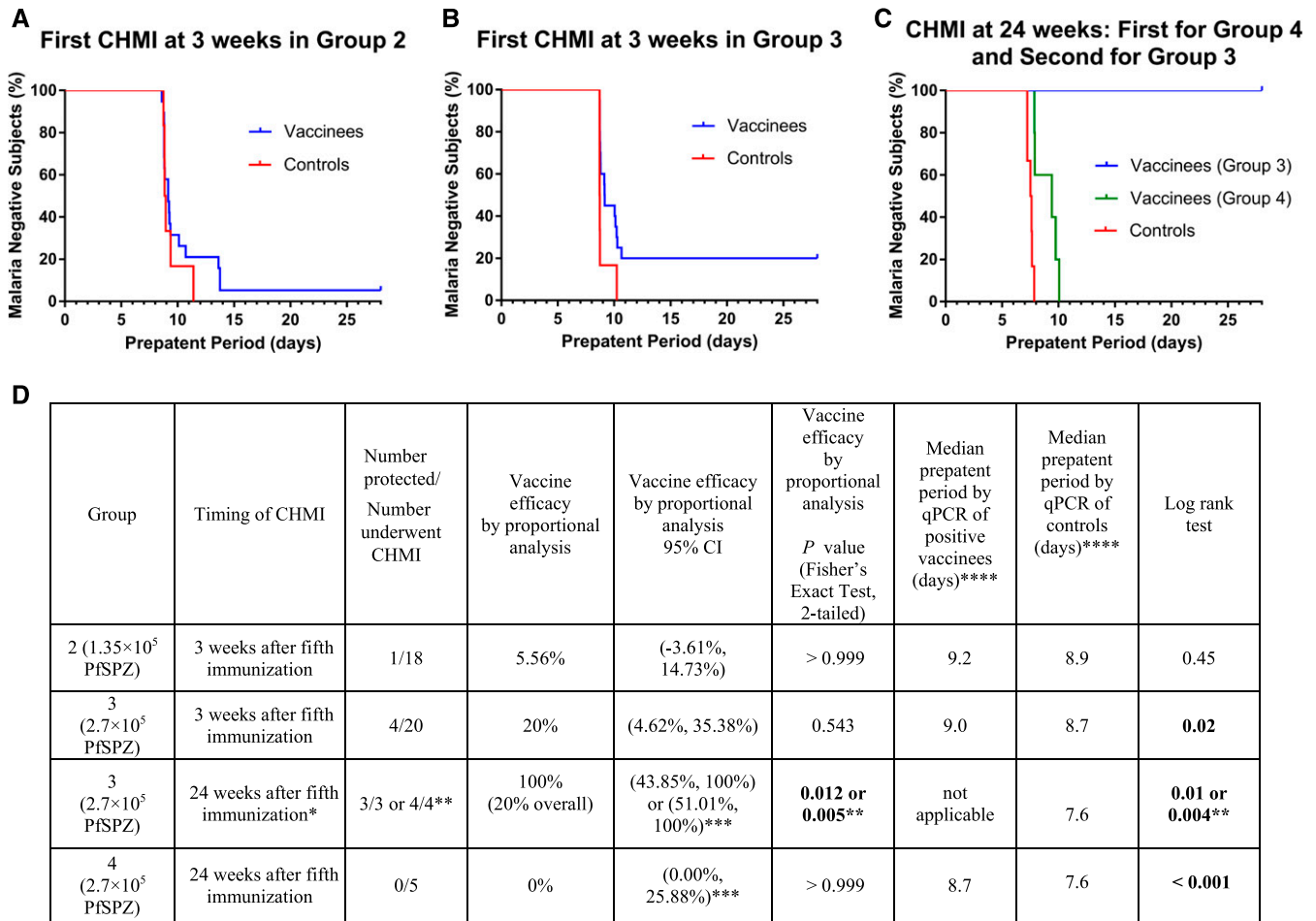


FIGURE 2. Kaplan–Meier survival curves in immunized volunteers vs. controls as assessed by quantitative polymerase chain reaction (qPCR). Kaplan–Meier curves in volunteers undergoing controlled human malaria infection (CHMI) 3 weeks after the last of five doses with  $1.35 \times 10^5$  (Group 2) (A) or  $2.7 \times 10^5$  (Group 3) (B) *Plasmodium falciparum* Sporozoites (PfSPZ) of PfSPZ Vaccine. Panel (C) volunteers undergoing either first (Group 4) or second (Group 3) CHMI 24 weeks after the fifth immunization with  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine. (D) Vaccine efficacy and prepatent period results. \*This was the second CHMI for the 4 volunteers in Group 3 who were protected after the first CHMI at 3 weeks. \*\*One volunteer was inappropriately treated on day 13 for a false positive TBS. Without this volunteer, 3/3 protected. With this volunteer 4/4 were protected. \*\*\*Confidence intervals were calculated using Wilson's score interval. \*\*\*\*Volunteers in CHMI #1 and #2 (3 week CHMI in Groups 2 and 3) had specimens first acquired on day 9. Volunteers in CHMI #3 (24 week CHMI in Groups 3 and 4) had specimens first acquired on day 8. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

**Prepatent periods and parasite densities.** Although the median prepatent periods by TBS in controls in each CHMI group (12.5, 13.0, and 12.0, respectively) were shorter than in the vaccinees in Groups 2–4 (14.0, 14.0, and 15.3 days, respectively), these differences did not reach the level of statistical significance ( $P = 0.486$ ,  $P = 0.491$ , and  $P = 0.333$ , respectively) (Supplemental Table 9). The prepatent periods by qPCR in vaccinees in Group 3 (3 and 24 week CHMIs) and Group 4 (24 week CHMI) were significantly longer than in the respective controls (Figure 2D). The parasite densities by qPCR and TBS at the time of diagnosis for each individual are in Supplemental Table 10. The median parasite density in controls versus vaccinees at the time of first positivity were 0.5 versus 0.4 parasites/ $\mu$ L for qPCR ( $P = 0.5714$ ) and 11.2 versus 15.0 parasites/ $\mu$ L for TBS ( $P = 0.1492$ ).

**Tolerability and safety of parasitemia during CHMI.** Controls. Sixteen controls developed parasitemia by TBS; 9 (56%) never had symptoms (Supplemental Table 11). Headache occurred in 7/7 symptomatic individuals. One of two control volunteers only positive by qPCR did not have any symptoms;

the second had headache 8 days after qPCR spontaneously reverted to negative. No volunteer had symptoms at the time of first positive qPCR.

**Vaccinees.** Thirty-five immunized volunteers developed parasitemia by TBS; 20 (57%) never had symptoms. Three volunteers had temperature  $> 39.0^\circ\text{C}$ ; all other clinical manifestations were grade 1 or 2. Fever (28.6%) and headache (31.4%) were most common. Compared with controls, elevated temperature was more common in vaccinees with positive TBSs (9/35 versus 0/16,  $P = 0.043$ ). There was no significant difference in the frequency of headache between controls and vaccinees. In the three volunteers in Group 2 who were qPCR positive and TBS negative, one developed headache 3 days after qPCR positivity. No volunteer had symptoms at the time of first positive qPCR.

**Clinical laboratories.** No unexpected changes were observed following CHMI. Declines in lymphocyte counts were observed in TBS positive controls and vaccinees (mean decline  $1,110 \pm 720$  cells/ $\mu$ L and  $1,180 \pm 680$  cells/ $\mu$ L, respectively) on day of first positive TBS. Absolute lymphocyte

counts less than 1,000 cells/ $\mu$ L were observed in 8/16 and 16/35 TBS positive controls and vaccinees. All lymphocyte counts returned to the baseline by day 28. There were mild decreases in platelet counts in TBS positive subjects, but all platelet counts were  $> 100 \times 10^3$  cells/ $\mu$ L.

**Treatment.** Volunteers with positive TBSs were treated with either atovaquone/proguanil ( $N = 43$ ) or artemether/lumefantrine ( $N = 8$ ) within 24 hours of first positive TBS. Normal saline and infectivity controls who were TBS negative ( $N = 2$ ) were treated at day 28.

**Immunogenicity. Antibody responses.** Pf circumsporozoite protein (PfCSP) and PfSPZ. Antibodies against PfCSP by ELISA 1), PfSPZ by aIFA 2), and PfSPZ by ISI 3) in sera taken 2 weeks after the last vaccine dose and just before CHMI (20–23 days after the last dose) for Groups 2 (CHMI #1) and 3 (CHMI #2) are in Figure 3A–C. The median responses and those uninfected and infected by qPCR are shown.

For all three assays, median antibody responses before first CHMI were higher in uninfected than in infected vaccinees. There was a significant difference in median net aIFA

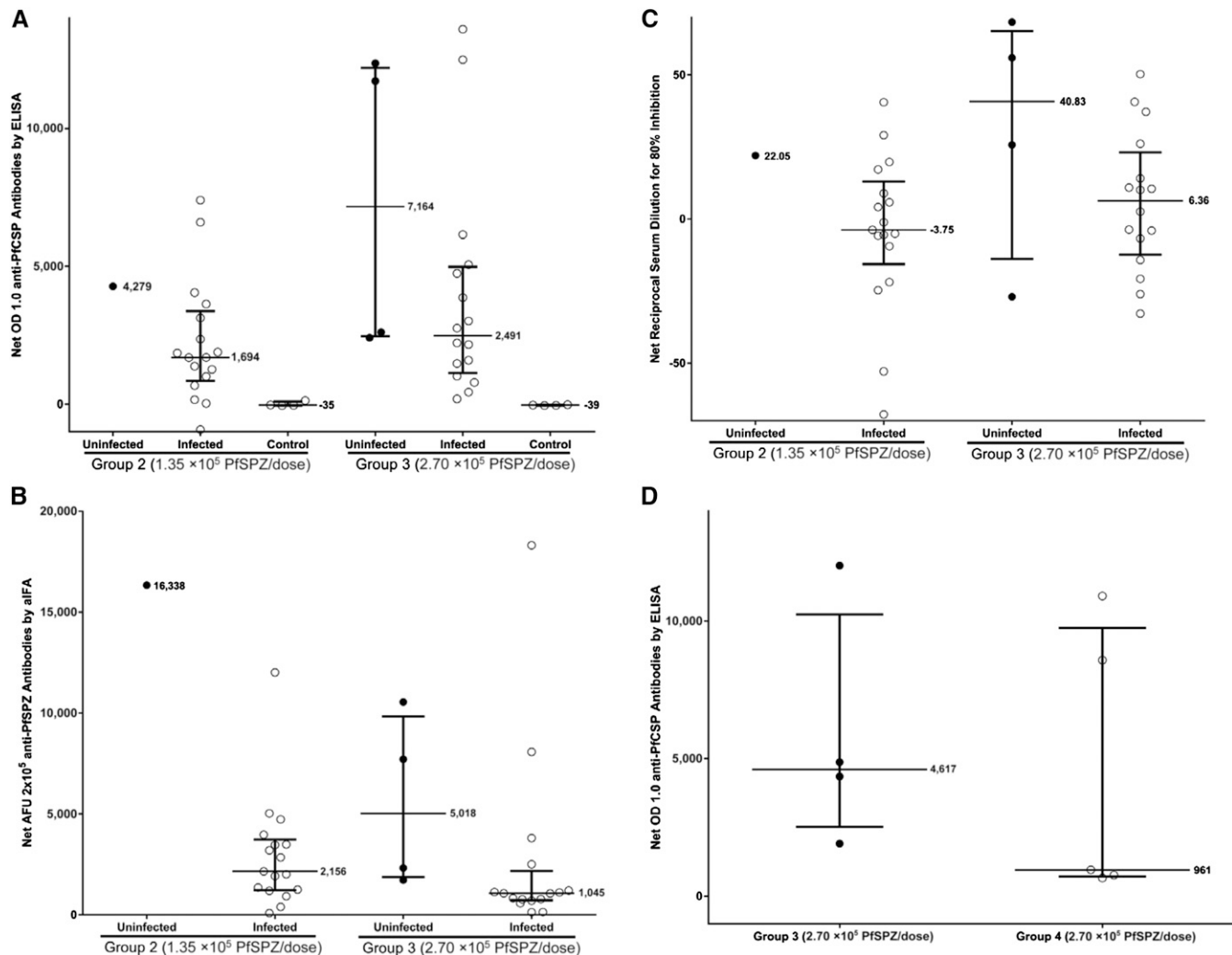


FIGURE 3. Antibody responses to *Plasmodium falciparum* Sporozoites (PfSPZ) and PfCSP before controlled human malaria infection (CHMI). For all assays, uninfected subjects are shown as filled (black) circles and infected subjects are open circles. For each of the defined subject groups, the interquartile ranges and the median values of response of subjects in each group are shown. Assessment of antibodies was performed in sera from subjects before immunization and before CHMI #1 (~2 weeks after the last dose of PfSPZ Vaccine or normal saline [NS]) and/or CHMI #2 (~24 weeks after last dose of PfSPZ or NS) (A, D). Antibodies to PfCSP by ELISA are reported as net optical density (OD) 1.0 (the difference in OD 1.0 between pre-CHMI and preimmunization sera). (B, E) Antibodies to PfSPZ by aIFA are reported as net AFU  $2 \times 10^5$ , the reciprocal serum dilution at which the fluorescent units were  $2 \times 10^5$  (AFU  $2 \times 10^5$ ) in pre-CHMI minus preimmunization sera. (C, F) Results of inhibition of sporozoite invasion (ISI) assay are reported as serum dilution at which there was 80% reduction of the number of PfSPZ that invaded a human hepatocyte line (HC-04) in the presence of pre-CHMI as compared with preimmunization sera from the same subject. Panels A–C show groups 2 (five doses of  $1.35 \times 10^5$  PfSPZ) and 3 (five doses of  $2.7 \times 10^5$  PfSPZ) before short-term CHMI (2 weeks after the last dose of PfSPZ or NS) and panels D–F show those volunteers in Groups 3 (five doses of  $2.7 \times 10^5$  PfSPZ) and 4 (five doses of  $2.7 \times 10^5$  PfSPZ) who underwent long-term CHMI (24 weeks after the last dose of PfSPZ). Panel G shows net optical density (OD) 1.0 anti-PfCSP antibodies by an enzyme-linked immunosorbent assay (ELISA) comparing vaccinated Tanzanian volunteers to volunteers in other trials receiving the same regimen. After five doses of  $2.7 \times 10^5$  PfSPZ/dose, volunteers in bagamoyo sporozoite vaccine 1 (BSPZV1) ( $N = 25$ ) had a 4.3-fold lower median net OD 1.0 than those in the U.S.-based clinical trial Walter Reed Army Institute of Research (WRAIR) 2080 ( $N = 26$ ) but a 6.6-fold higher median OD 1.0 than volunteers in 14-I-N010 in Bamako, Mali ( $N = 42$ ), where malaria transmission rates are higher. There was a significant difference between the results for WRAIR 2080 vs. BSPZV1 ( $P = 0.0012$ ), WRAIR 2080 vs. 14-I-N010 ( $P < 0.0001$ ), and even 14-I-N010 vs. BSPZV1 ( $P = 0.002$ ) (two-tailed  $t$ -test). AFU = arbitrary fluorescence units; aIFA = antibodies by immunofluorescence assay.

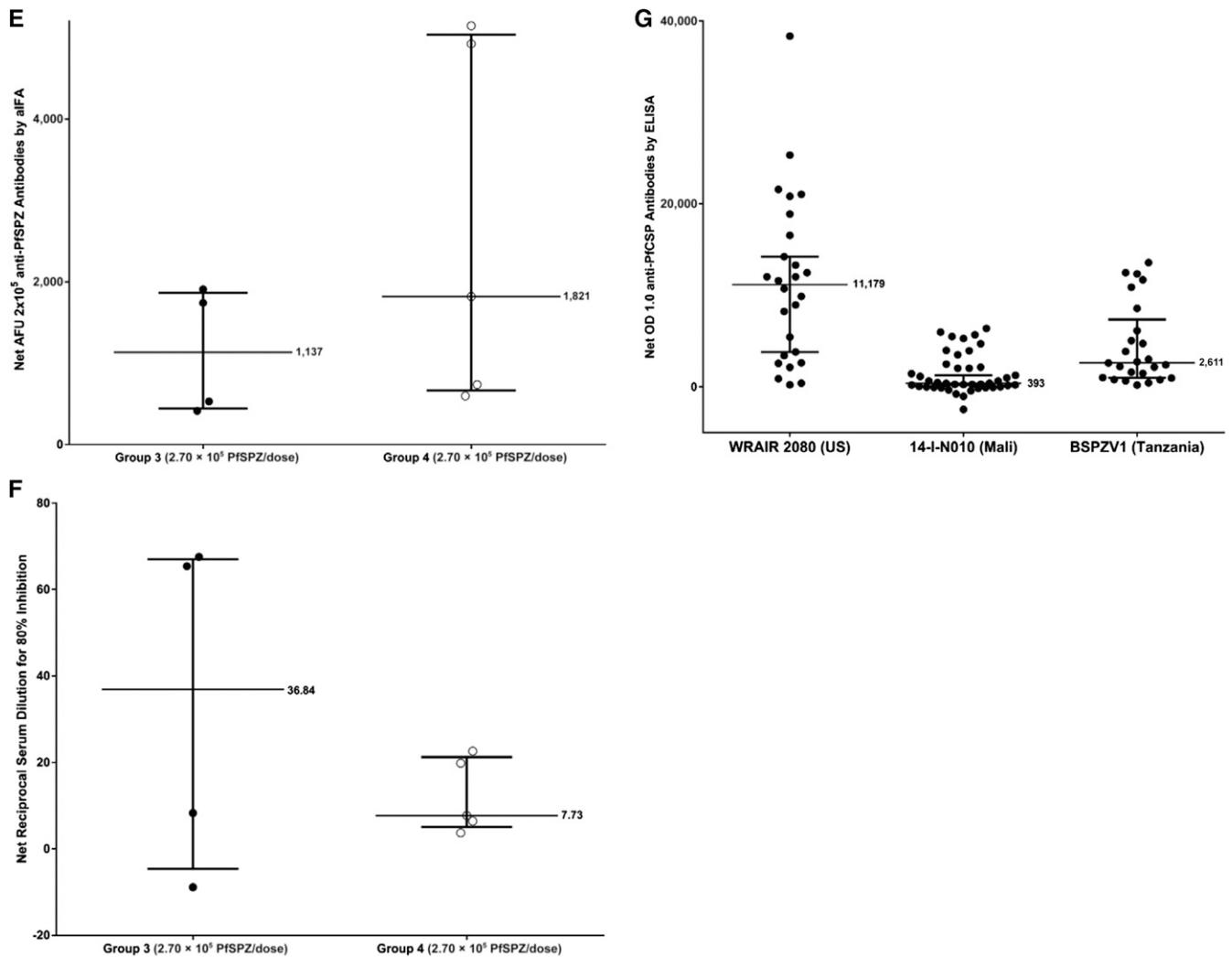


FIGURE 3. (Continued)

responses between infected and uninfected volunteers in Group 3 before CHMI #1 ( $P = 0.0499$ , Wilcoxon Rank-Sum Test), but not PfCSP ( $P = 0.290$ ) or for ISI ( $P = 0.249$ ).

In sera collected before CHMI #3 (170–171 days after the last vaccine dose), antibodies by the three assays for Group 4 and for the four volunteers in Group 3 uninfected in CHMI #1 who underwent CHMI #2 are in Figure 3D–F. All data appear in Supplemental Table 12.

After the fifth dose, in the PfCSP ELISA, volunteers were considered to have seroconverted if their net OD 1.0 and OD 1.0 ratio calculated, respectively, by subtracting or dividing by the prevaccination antibody OD 1.0, were  $\geq 50$  and  $\geq 3.0$ . By these criteria, 15/18 volunteers (83%) in Group 2, 20/20 (100%) in Group 3, and 5/5 (100%) in Group 4 seroconverted, median net OD 1.0 of positives of 1,189, 2,685, and 961, and median OD 1.0 ratio of positives of 11.50, 21.15, and 37.83, respectively (Supplemental Table 13). In the aIFA, volunteers with a net arbitrary fluorescence unit (AFU)  $2 \times 10^5$  of  $\geq 150$  and a ratio of post- to pre-AFU  $2 \times 10^5$  of  $\geq 3.0$  were considered positive (Supplemental Table 13). By these criteria, 17/18 volunteers (94%) in Group 2, 18/20 (90%) in Group 3, and 5/5 (100%) in Group 4 seroconverted, median net OD 1.0 of positives of 2,844, 1,165, and 1,820, and median OD 1.0 ratio

of positives of 1,193.00, 552.88, and 224.86, respectively (Supplemental Table 13). For the ISI, volunteers with a net ISI activity of  $\geq 10\%$  and a ratio of post- to pre-ISI activity of  $\geq 3.0$  were considered positive. By these criteria, 3/18 volunteers (17%) in Group 2, 8/20 (40%) in Group 3, and 3/5 (60%) in Group 4 were positive, median net OD 1.0 of positives of 22.05, 38.92, and 12.44, and median OD 1.0 ratio of positives of 19.79, 12.53, and 13.44, respectively (Supplemental Table 13).

**Other antigens.** Two weeks after the fifth dose in Group 2 ( $1.35 \times 10^5$  PfSPZ) and groups 3 and 4 ( $2.7 \times 10^5$  PfSPZ), there were antibodies to PfCSP in 15/18 and 25/25 subjects, respectively. Ten of 25 volunteers immunized with  $2.7 \times 10^5$  PfSPZ made antibodies to Pf apical membrane antigen 1 and 4–16% responded to PfCelTOS, PfMSP5, PfMSP1, or Pf erythrocyte binding antigen 175 (PfEBA175) (Supplemental Table 14). The presence of antibodies, albeit at low incidence, against proteins first expressed in late liver stages (PfMSP1 and PfEBA175) was unexpected; results were confirmed by repeating the assays. No antibody responses were associated with protection.

**T-cell responses.** T cells against liver-stage malaria parasites in mice and nonhuman primates immunized with

radiation-attenuated SPZ mediate protection<sup>9,29–31</sup> and it is likely this is the case in humans.<sup>12</sup> CD8 and CD4 T-cell responses generally peak after the first vaccination with PfSPZ Vaccine.<sup>13</sup> In this trial, T-cell responses were measured before immunization, 2 weeks after the first and 2 weeks after the final immunization in Group 2 ( $1.35 \times 10^5$  PfSPZ). For technical reasons (loss of viability), the other groups could not be studied.

After the first vaccination, the percent of Pf red blood cell (PfRBC)-specific and PfSPZ-specific cytokine-producing memory CD4 T-cell responses increased by  $0.25 \pm 0.06$  (mean  $\pm$  SEM) and  $0.24 \pm 0.04$ , respectively (Figure 4A, B). Throughout, “naïve T cell” refers to cells that co-express CCR7 and CD45RA, and “memory T cell” refers to all other T cells. After the final vaccination, at week 22, the CD4 T-cell responses were above prevaccine responses by  $0.17 \pm 0.05$  and  $0.18 \pm 0.05\%$  points, respectively. These responses were lower than after the same immunization regimen in malaria-naïve U.S. adults.<sup>10</sup>

PfRBC-specific CD8 T cells were not significantly above the prevaccine levels, and PfSPZ-specific CD8 T cells were slightly above background (Figure 4C, D); responses were lower than in U.S. adults.<sup>10,12</sup>

In contrast to other PfSPZ Vaccine trials,<sup>10,12–14</sup> there was negligible change in the frequency of circulating  $\gamma\delta$  T cells (Figure 4E) or activation as measured by change in expression of the activation markers HLA-DR and CD38 following immunization (Figure 4F). To identify potential explanations for lower cellular immune responses in Tanzanians, we examined frequency of T regulatory (Treg) cells ( $CD4^+Foxp3^+CD25^+CD127^-$ ) expressing the activation marker CD137 (also known as 4-1BB)<sup>32</sup> after stimulation with PfRBC. There was no difference in prevaccine frequency of PfRBC-specific Tregs in the Tanzanians as compared with Americans<sup>10</sup> (Figure 4G). Consistent with CD4 and CD8 T-cell responses, PfRBC-specific Tregs were highest after first immunization (Figure 4H). Last, the prevaccine frequency of total memory T cells relative to total naïve T cells was significantly higher than in Americans (Figure 4I).

## DISCUSSION

To our knowledge, this was the first assessment of the VE of a malaria vaccine in Africa against CHMI. *Plasmodium falciparum* SPZ Vaccine was well tolerated and safe but less immunogenic and protective in Tanzanian men than in U.S. volunteers.

In our studies, all 18 controls became infected. Four of 20 (20%) recipients of five doses of  $2.7 \times 10^5$  PfSPZ did not become infected after homologous CHMI by DVI 3 weeks after the last immunization. By contrast, 12/13 (92.3%) volunteers in the United States who received five doses of  $2.7 \times 10^5$  PfSPZ were protected after homologous CHMI by mosquito bite 3 weeks after the last vaccine dose.<sup>11</sup> When the four uninfected Tanzanian volunteers underwent repeat homologous CHMI at 24 weeks after the last dose, all four (100%) were protected. In the United States, Seven of 10 previously protected volunteers were protected when they underwent homologous CHMI at 24 weeks<sup>11</sup> and all five volunteers in the United States who were protected at 21 weeks after the last immunization (four doses of  $2.7 \times 10^5$  PfSPZ) were protected against repeat mosquito-administered CHMI at 59 weeks.<sup>12</sup> This could be due to boosting by the small numbers of PfSPZ administered during the CHMI, or is more likely due to the fact

that in these protected individuals, the protective immune responses induced by immunization were sustained.

The same exact immunization regimen was assessed for VE against intense field transmission of heterogeneous Pf in Mali. Vaccine efficacy against infection with Pf on TBS was 52% by time to event and 29% by proportional analysis during 24 weeks after the last vaccine dose.<sup>14</sup> This was higher than the VE by proportional analysis against homologous CHMI in Tanzania. In Tanzania, there was a significant delay in the onset of parasitemia after CHMI at 3 and 24 weeks in subjects who received five doses of  $2.7 \times 10^5$  PfSPZ and were not fully protected (Figure 2B–D). Nonetheless, the proportional analysis suggests that homologous CHMI by DVI of a 100% infectious dose of homologous PfSPZ Challenge is at least as rigorous as a test of VE and potentially more rigorous than intense field transmission of heterogeneous Pf.

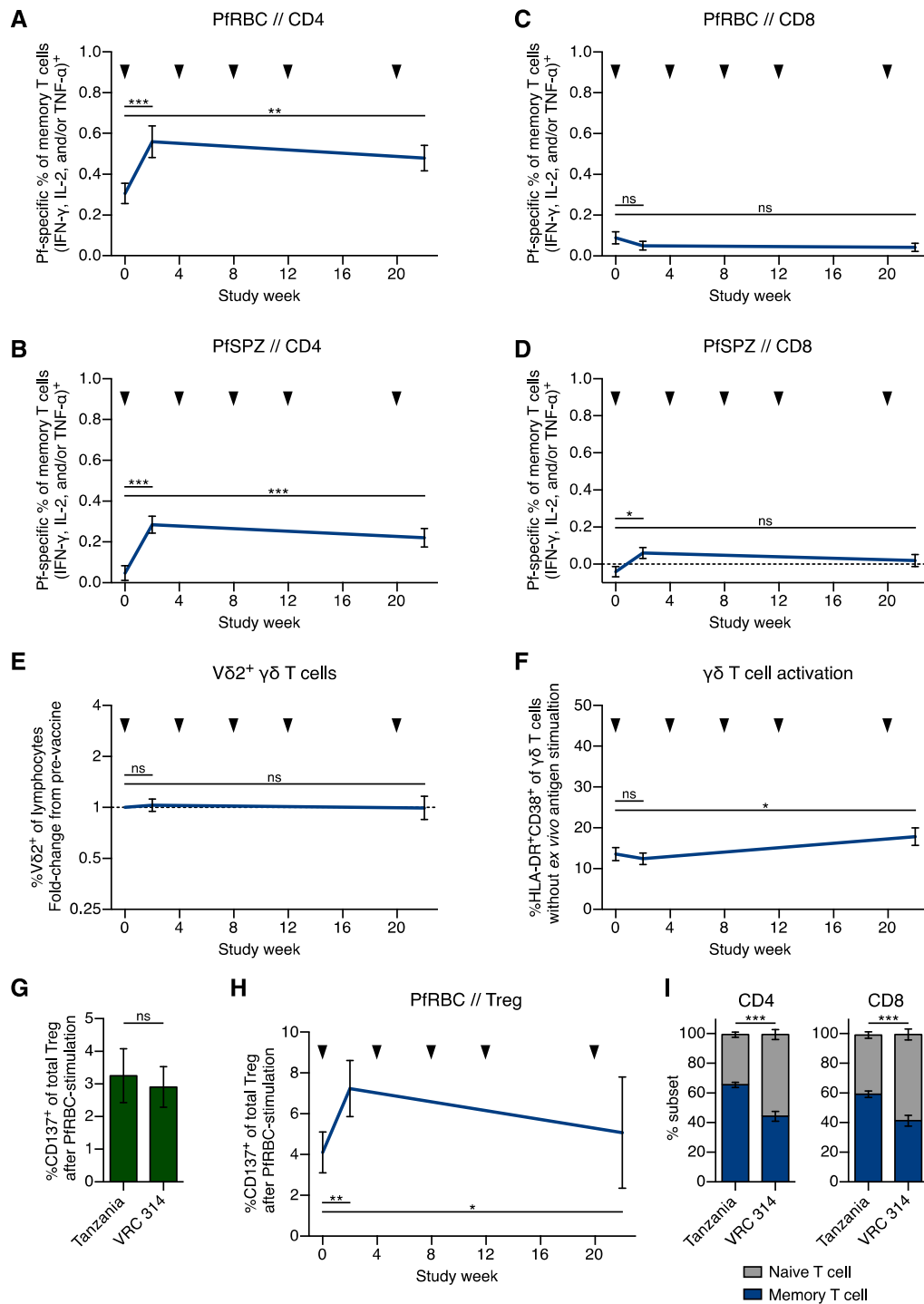
Vaccine-induced antibody and T-cell responses in the Tanzanians were lower than in malaria-naïve Americans who received the exact same dosage regimen. Two weeks after the last dose, the median antibody responses to PfCSP, the major protein on the surface of PfSPZ, were 4.3 times lower in the Tanzanians than those in Americans ( $P = 0.0012$ , Student's *t*-test, 2-tailed),<sup>11</sup> but significantly higher than in Malians who received the same immunization regimen ( $P = 0.002$ )<sup>14</sup> (Figure 3G).

The T-cell responses were also lower than in Americans<sup>10,12</sup> (Figure 4), but this could only be assessed in PBMCs from individuals who received the lower dose (five doses of  $1.35 \times 10^5$  PfSPZ), not in the individuals who received the higher dose (five doses of  $2.7 \times 10^5$  PfSPZ), the group that had sustained protection for 24 weeks. Thus, it is possible that had PBMCs from the higher dose group been assessed, responses would have been comparable to the responses in nonimmune Americans. The Tanzanians who were assessed had a significantly higher proportion of total memory T cells compared with total naïve T cells at the baseline than did the Americans. This higher frequency of memory cells compared with naïve cells may explain the lower immunogenicity due to less available naïve cells for expansion during the vaccinations. Moreover, the greater frequency of non-Pf-specific memory T cells may compete for infected cell contacts during pathogen surveillance.<sup>33</sup> These data suggest that PfSPZ Vaccine immunogenicity may be dependent on cumulative history of Pf exposure. Another explanation is that an activated immune microenvironment in the Tanzanians as compared with the Americans reduced immune responses.<sup>34</sup> Helminth infections have been associated with reduced immune responses to malaria<sup>35</sup>; however, the paucity of helminth infections in this population does not support helminth infection as a cause of the reduced immune responses.

There were no differences between vaccine and NS placebo recipients in regard to vaccine tolerability or AEs; 97.1% of the DVI administrations were rated painless and no volunteer experienced any local AE. Systemic AEs, most commonly headache, were mild, infrequent, and of short duration, with a similar frequency in NS controls as in vaccinees (no statistically significant differences in rates).

Among the controls, 16 of 18 were positive for Pf by TBS after CHMI. However, all 18 were positive by qPCR. This is consistent with findings in Gabon after CHMI.<sup>21</sup> It is likely that preexisting asexual blood stage immunity limits Pf replication in some individuals. Thus, they never reach the threshold for detection by





**FIGURE 4.** *Plasmodium falciparum* Sporozoites (PfSPZ)-specific T-cell responses in vaccine recipients receiving  $1.35 \times 10^5$  PfSPZ. **(A–D)** PfSPZ-specific T-cell responses. Frequency of cytokine-producing memory CD4 T cells responding to **(A)** PfRBC or **(B)** PfSPZ. Throughout, “naive T cell” refers to cells that co-express CCR7 and CD45RA, and “memory T cell” refers to all other T cells. Frequency of cytokine-producing memory CD8 T cells responding to **(C)** PfRBC or **(D)** PfSPZ. Results are the percentage of memory T cells producing interferon gamma, interleukin 2, and/or tumor necrosis factor alpha following stimulation minus the percentage of cells following control stimulation. **(E)** Frequency of the Vδ2<sup>+</sup> subfamily of γδ T cells of total lymphocytes. Results are expressed as fold-change from the prevaccine frequency. **(F)** γδ T-cell activation in vivo. Data are the percentage of memory γδ T cells expressing HLA-DR and CD38 as measured on PBMCs following incubation with control stimulation (vaccine diluent). **(G)** Prevaccine frequency of PfRBC-specific Tregs in Tanzania compared with malaria-naïve U.S. subjects from the Vaccine Research Center (VRC) 314 study. **(H)** Frequency of PfRBC-specific Treg. Results are the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup>CD25<sup>+</sup>CD127<sup>−</sup> T cells expressing CD137 (also known as 4-1BB) after stimulation with Pf red blood cell (PfRBC) minus the percentage of cells following stimulation with uninfected RBC. **(I)** Percentage of total CD4 (left) or CD8 (right) T cells that are naïve (gray bar; CCR7<sup>+</sup>CD45RA<sup>+</sup>) or memory (blue bar; not CCR7<sup>+</sup>CD45RA<sup>+</sup>) phenotype assessed prevaccination in all 48 subjects vaccinated in Tanzania or in 14 healthy U.S. subjects from the VRC 314 study.<sup>13</sup> For **A–F** and **H**,  $N = 24$ , and statistical difference was measured by using the Wilcoxon matched-pairs signed rank test. For **G** and **I**, statistical difference was measured by using the Mann–Whitney  $U$  test.  $P$  values are reported as not significant (ns),  $< 0.05$  (\*),  $< 0.01$  (\*\*), or  $< 0.001$  (\*\*\*). Data are mean  $\pm$  SEM. Time points are prevaccine, 2 weeks after the first vaccination, and 2 weeks after the final vaccination. Black arrowhead designates PfSPZ Vaccine administration. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

TBS. In our CHMI studies in Bagamoyo, we now use qPCR to confirm positive TBS, and retrospectively or in real time, assess parasitemia in all volunteers by qPCR.

We propose that increasing the numbers of PfSPZ per dose and altering intervals between doses will lead to overcoming the downregulation of humoral and cell-mediated immunity most likely because of previous exposure to Pf and thereby increase immune responses to PfSPZ Vaccine and VE. We also hypothesize that immune responses in younger, less malaria-exposed individuals will be of greater magnitude than those in adults.

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# Advancing Global Health through Development and Clinical Trials Partnerships: A Randomized, Placebo-Controlled, Double-Blind Assessment of Safety, Tolerability, and Immunogenicity of PfSPZ Vaccine for Malaria in Healthy Equatoguinean Men

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**Abstract.** Equatorial Guinea (EG) has implemented a successful malaria control program on Bioko Island. A highly effective vaccine would be an ideal complement to this effort and could lead to halting transmission and eliminating malaria. Sanaria® PfSPZ Vaccine (*Plasmodium falciparum* sporozoite Vaccine) is being developed for this purpose. To begin the process of establishing the efficacy of and implementing a PfSPZ Vaccine mass vaccination program in EG, we decided to conduct a series of clinical trials of PfSPZ Vaccine on Bioko Island. Because no clinical trial had ever been conducted in EG, we first successfully established the ethical, regulatory, quality, and clinical foundation for conducting trials. We now report the safety, tolerability, and immunogenicity results of the first clinical trial in the history of the country. Thirty adult males were randomized in the ratio 2:1 to receive three doses of  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine ( $N = 20$ ) or normal saline placebo ( $N = 10$ ) by direct venous inoculation at 8-week intervals. The vaccine was safe and well tolerated. Seventy percent, 65%, and 45% of vaccinees developed antibodies to *Plasmodium falciparum* (Pf) circumsporozoite protein (PfCSP) by enzyme-linked immunosorbent assay, PfSPZ by automated immunofluorescence assay, and PfSPZ by inhibition of sporozoite invasion assay, respectively. Antibody responses were significantly lower than responses in U.S. adults who received the same dosage regimen, but not significantly different than responses in young adult Malians. Based on these results, a clinical trial enrolling 135 subjects aged 6 months to 65 years has been initiated in EG; it includes PfSPZ Vaccine and first assessment in Africa of PfSPZ-CVac. ClinicalTrials.gov identifier: NCT02418962.

## INTRODUCTION

Malaria has major public health significance in sub-Saharan Africa.<sup>1</sup> World Health Organization-Global Technical Strategy has set a goal of eliminating malaria from at least 10 malaria-endemic countries by the year 2020.<sup>2</sup> Development and deployment of new tools such as highly efficacious malaria vaccines that can interrupt malaria transmission will be essential to achieve this goal. The RTS,S/AS01 candidate malaria vaccine has shown moderate efficacy against clinical disease in young children,<sup>3</sup> but its limited efficacy against infection and significant adverse events (AEs) restrict its usefulness for malaria elimination.<sup>4,5</sup>

Sanaria® PfSPZ Vaccine is a live attenuated *Plasmodium falciparum* (Pf) whole sporozoite (SPZ) vaccine that is currently being assessed in clinical trials in the United States, Europe, and Africa. PfSPZ Vaccine has been well tolerated, safe, and protective against controlled human malaria infection (CHMI) in the United States and against natural exposure to malaria in Mali.<sup>6,7</sup> Protection durable for at least 6–14 months has been demonstrated.<sup>7–10</sup>

A robust malaria control program has been in place on Bioko Island, Equatorial Guinea (EG) since 2004.<sup>11</sup> Using insecticide-treated bed nets, indoor residual spraying and early diagnosis

and treatment with artemisinin combination therapy,<sup>11</sup> the Bioko Island Malaria Control Program has reduced the average prevalence of malaria parasitaemia in the island's children from 45% in 2000 to 11.1% in 2016. To capitalize on this success, the EG Malaria Vaccine Initiative (EGMVI) was established in 2014 to assess the utility of PfSPZ Vaccine to eliminate malaria. EGMVI is funded by the Government of EG and three U.S. energy companies, Marathon EG Production Limited, Noble Energy, Atlantic Methanol Production Company, and EG LNG, and aims to culminate its activities with an island-wide mass vaccination program.

Here we report the findings from the first clinical trial in the history of EG. This was a phase 1 clinical trial to evaluate the safety, tolerability, and immunogenicity of three doses of  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine administered by direct venous inoculation (DVI) to healthy, malaria-exposed semi-immune young Equatoguinean men living in a malaria-endemic region of Bioko Island, EG.

## MATERIALS AND METHODS

**Clinical trial design.** The primary objective of this study was to assess the safety, tolerability and immunogenicity of three doses of  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine as compared with normal saline (NS) placebo administered to healthy young Equatoguinean men by DVI at 8-week intervals. The study was conducted at La Paz Medical Center, Malabo, EG. A Safety Monitoring Committee (SMC) was appointed by the study sponsor and consisted of three external experts and the local

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safety monitor, who was an independent Equatoguinean physician based in Malabo and accessible during the entire study period.

The study had three groups with a total of 33 volunteers. Group 1, the sentinel safety group, included three volunteers who received  $1.35 \times 10^5$  PfSPZ followed by  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine 2 weeks later. The SMC reviewed the data from Group 1 and then recommended proceeding with vaccination of the rest of the volunteers.

For Groups 2 and 3, the study was a phase 1 randomized, double-blind, placebo-controlled clinical trial. Twenty volunteers in Group 2 received three doses of  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine and 10 volunteers in Group 3 received three doses of NS at 8-week intervals.

**Ethical considerations.** Ethical approval was obtained from the National Ethics Committee of EG, the Ifakara Health Institute Institutional Review Board, Dar es Salaam, Tanzania, the McGill Institutional Review Board in Maryland, USA, and the Ethical Review Committee of Northwestern and Central Switzerland. The study was registered at ClinicalTrials.gov (ClinicalTrials.gov registration number: NCT02418962). The volunteers were briefed on the specifics of the planned study, and then were assessed for their understanding of study procedures using a multiple-choice questionnaire. Only those who correctly answered all 10 questions proceeded with the consenting procedure. All volunteers gave a written informed consent before any study procedure was done. As EG is a Spanish-speaking country, approved Spanish-translated informed consent forms were used during the consenting process, which was conducted by investigators who were fluent in Spanish.

**Vaccine.** PfSPZ Vaccine is composed of aseptic, purified, metabolically active, nonreplicating (live, radiation attenuated) cryopreserved PfSPZ.<sup>12,13</sup> The vaccine is stored in liquid nitrogen vapor phase at  $-150^\circ\text{C}$  to  $-196^\circ\text{C}$ . Before administration, PfSPZ Vaccine is thawed and then diluted in phosphate-buffered saline (PBS) with human serum albumin to achieve the correct dose.

**Study population and enrollment criteria.** Volunteers were healthy male adults between 18 and 35 years of age recruited from the towns of Baney and Rebola located in Baney District, Bioko Island. The study area has low malaria endemicity with estimated prevalence in 2016 of less than 1% in children aged 2 to 14 years. Volunteers were excluded from enrollment if they were taking immunosuppressive drugs, or had 1) clinically significant acute or chronic diseases, 2) clinically significant abnormalities in electrocardiogram at screening, 3) plans to travel outside Bioko Island, EG, in the first 9 months of the study, 4) immunosuppressive disorders, 5) a positive blood test for hepatitis B surface antigen (HBsAg), hepatitis C virus, or human immunodeficiency virus (HIV)-1, or 6) clinically significant abnormalities on basic hematology, hepatic and renal function laboratory tests. Volunteers with confirmed malaria parasitemia by microscopy of Giemsa-stained thick blood smears (sensitivity of 2–4 parasites/ $\mu\text{L}$  blood), intestinal parasites by microscopy of Lugol-stained fecal material (10 $\times$  objective for detection and quantification and 40 $\times$  objective for species identification), or urinary tract infections were treated and confirmed cured before they were enrolled into the study. In addition, volunteers with risk factors for clinically active tuberculosis plus a positive tuberculin skin test were excluded.

**Randomization and vaccination.** No randomization was performed in Group 1 in which all volunteers received PfSPZ Vaccine. Participants in Group 2 and 3 were randomized in a final ratio of 2:1 to receive three doses of either  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine or NS. Randomization was done using a computer-generated list of random numbers by an independent statistician from the Swiss Tropical and Public Health Institute. At enrollment, volunteers were assigned either PfSPZ Vaccine or NS as specified in a master randomization list.

PfSPZ Vaccine and NS were administered as a 0.5 mL volume by DVI using a 1 mL syringe with a 25-gauge needle. Syringe preparation was done in a biological safety cabinet in a pharmacy out of sight of the study physicians and nurses who evaluated the study end points and of the study volunteers. Pharmacy personnel preparing the doses were aware of the treatment assignments, but took no other part in study-related procedures and were instructed not to reveal the assignments to either the volunteers or study investigators. The study clinical team was blinded to treatment.

**Definitions.** An AE was defined as any unfavorable or unintended change in body structure, body function or laboratory result associated temporally with the study treatment, whether or not it was considered to be related to the study treatment. A serious AE (SAE) was defined as any untoward medical occurrence that resulted in death, was life threatening, required hospitalization or prolongation of hospitalization, resulted in disability, or was otherwise considered serious by the investigators. All AEs were classified according to the preferred term in the Medical Dictionary for Regulatory Activities (MedDRA). When the report filed by the investigator did not clearly correspond to a MedDRA term, the most appropriate classification was clarified in discussion between the study sponsor and the investigators before unblinding.

**Outcome measures.** The primary outcome measures were the occurrence of solicited and unsolicited AEs during a 7-day and 28-day surveillance period after each vaccination, respectively. SAEs were collected throughout the study period, which extended 24 weeks after the last dose of study product. Local solicited AEs included erythema, swelling, induration, pain, and tenderness at the injection site. Systemic solicited AEs included malaise, nausea, vomiting, abdominal pain, diarrhea, fever (axillary temperature  $\geq 37.5^\circ\text{C}$ ), arthralgia, myalgia, fatigue, chills, headache, chest pain/discomfort, shortness of breath, palpitations, and potential allergic reactions (wheezing, urticaria, edema/angioedema, hypotension, anaphylaxis). All solicited local AEs were considered definitely related to the study product and solicited systemic AEs were considered to be possibly or probably related because of existence of alternative causes.

**Safety monitoring.** Before being vaccinated, all volunteers were trained to measure their body temperature using digital thermometers. After each vaccination, the volunteers were evaluated for solicited AEs at 15 minutes, 1 hour, and 2 hours. After 2 hours, the volunteers were transported to their homes where study personnel collected GPS coordinates of their houses. These were kept in the volunteers' files to facilitate follow-up in case of a missed scheduled visit. All volunteers were evaluated clinically at the study center 2, 7, 14, and 28 days after each vaccination, and every 4 weeks for 24 weeks after the last vaccination. In addition, study physicians called

the volunteers by telephone on days 1, 3, 4, 5, and 6 after each vaccination to solicit local and systemic AEs. All volunteers were provided with mobile phones and encouraged to come to the study center or call the study physician when they felt unwell. Study physicians evaluated symptoms identified at scheduled visits and appropriate management was provided. In case further care was required, volunteers were referred to appropriate services at La Paz Medical Center where high-quality inpatient and outpatient care was available 24 hours a day, 7 days a week. Clinical evaluations consisted of measurement of vital signs and assessment for local injection site and general solicited signs or symptoms. Blood for safety analyses was collected at screening, before each vaccination and on days 2, 7, 14, and 28 after each vaccination, and every 4 weeks for 24 weeks after the last vaccination. At screening, a complete blood count and serum biochemistry (alanine aminotransferase [ALT] and aspartate aminotransferase [AST], creatinine, bilirubin, potassium, sodium, and alkaline phosphatase) were assessed. At subsequent scheduled visits, a complete blood count and limited biochemistry (ALT, AST, and creatinine) were assessed. Biochemistry tests were performed using a Pentra 60C+ and hematological tests were done with a Roche Cobas Integra 400 Plus.

Solicited local AEs were assessed as mild, moderate, severe, or life threatening (grades 1, 2, 3, and 4, respectively). Pain at the injection site was categorized as grade 1: does not interfere with activity, grade 2: interferes with activity or requires repeated use of non-narcotic pain reliever > 24 hour, grade 3: prevents daily activity or requires any use of narcotic pain reliever, or grade 4: requires hospitalization. Tenderness was graded as grade 1: mild discomfort to touch, grade 2: discomfort with movement, grade 3: significant discomfort at rest, or grade 4: discomfort that requires hospitalization. Erythema, swelling, or induration at the injection site measured at greatest single diameter were grade 1: > 2.5–5 cm, grade 2: 5.1–10 cm, grade 3: > 10 cm, or grade 4: presence of necrosis. Solicited systemic AEs were assessed as mild, moderate, severe, or life threatening (grades 1, 2, 3, and 4 respectively), defined as grade 1: AEs that were easily tolerated; grade 2: AEs that interfered with daily activity, grade 3: AEs that prevented daily activity, or grade 4: AEs that required hospitalization.

The degree to which an AE could be attributed to vaccination was determined by the principal investigator (PI) with advice from the rest of the clinical team and categorized as not, unlikely, possibly, probably, or definitely related. For the final analysis categories, “not related” and “unlikely related” were combined into “unrelated.” “Possibly related,” “probably related,” and “definitely related” were combined into “related.” Abnormal laboratory findings were graded using an FDA-recommended<sup>14</sup> grading scheme adapted to be consistent with normal ranges in EG, Mali, and Tanzania. Abnormal laboratory tests were assessed as clinically significant if they were associated with clinical symptoms or required medical intervention or clinically nonsignificant if there were no associated symptoms, and they required no treatment. Information on any other symptoms, the use of any medications (prescription and/or over the counter), unscheduled medical consultations, and hospitalizations were also collected. All aspects of the trial were assessed by a sponsor-appointed clinical trial monitor, who also reviewed established safety stopping criteria together with the PI.

**Immunogenicity assessment.** Antibody responses were measured on sera obtained from participants before the first vaccination and 2 weeks after the last vaccination.

*IgG antibodies to P. falciparum circumsporozoite protein (PfCSP) by enzyme-linked immunosorbent assay (ELISA).* Antibodies against PfCSP were measured by ELISA at Sanaria Inc. Briefly, 96-well plates (Thermo Fisher Scientific, Rochester, NY) were coated overnight at 4°C with 2.0 µg/mL per well of recombinant PfCSP in 50 µL coating buffer (KPL - Sera Care, Milford, MA). Plates were washed three times with 2 mM imidazole, 160 mM NaCl, 0.02% Tween 20, 0.5 mM EDTA, and then blocked with 1% bovine serum albumin (BSA), blocking buffer (KPL - Sera Care, Milford, MA) containing 1% nonfat dry milk for 1 hour at 37°C. The plates were washed three times and serially diluted serum samples (in triplicates) were added and incubated at 37°C for 1 hour. After three washes, peroxidase-labeled goat antihuman IgG (KPL - Sera Care, Milford, MA) was added at a dilution of 0.1 µg/mL and incubated at 37°C for 1 hour. Plates were washed three times, ABTS peroxidase substrate was added for plate development, and the plates were incubated for 75 minutes at 22°C. The plates were read with a Spectramax Plus384 microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. The data were collected using Softmax Pro GXP v5 and fit to a 4-parameter logistic curve, to calculate the serum dilution at OD 1.0. A negative control (pooled serum from non-immune individuals from a malaria free area) was included in all assays. The positive control was pooled human sera taken 2 weeks after the last immunization from 12 volunteers immunized four or five times with PfSPZ Vaccine in the VRC 312 clinical trial, who did not develop parasitemia after CHMI.<sup>6</sup> Samples were considered positive if the difference between the post-immunization OD 1.0 and the pre-immunization OD 1.0 (net OD 1.0) was ≥ 50, and the ratio of post-immunization OD 1.0 to pre-immunization OD 1.0 was ≥ 3.

*IgG antibodies to PfSPZ by automated immunofluorescence assay (aIFA).* In the PfSPZ aIFA, the serum dilution at which the arbitrary fluorescence units (AFU) was  $2 \times 10^5$  was determined at Sanaria. Purified PfSPZ (NF54 strain) from aseptic *Anopheles stephensi* mosquitoes produced by Sanaria were resuspended in PBS (pH 7.4). Forty microliters containing  $0.5 \times 10^4$  PfSPZ were added to each well of Greiner cellstar clear bottom black 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany), the plates were then left at ambient temperature for 12–18 hour to air-dry. Two-fold serial dilutions (50 µL/well) of sera, starting at 1:50, diluted in PBS with 2% BSA were added to each well of the 96-well plate containing air-dried PfSPZ and incubated at 37°C for 1 hour. The plates were washed in PBS three times using an Aquamax Microplate washer. Alexa Fluor 488–conjugated goat antihuman IgG (Life Technologies Corporation, Eugene, OR) was diluted 1:250 in PBS with 2% BSA, and 40 µL were added to each well. The plates were then incubated for 1 hour at 37°C, washed three times with PBS, and then 100 µL PBS was added to each well. The plates were sealed using a plate sealer and stored in the dark at 4°C until data acquisition. Samples were assessed by scanning the plates using an Acumen eX3 laser scanning imaging cytometer. The positive control was the same pooled human sera used for ELISA. The Acumen image cytometer scans the entire surface area of each well in a 96-well plate, and the fluorescence intensity values (arbitrary

units) therefore represent the signal from all  $0.5 \times 10^4$  PfSPZ that were seeded in each well.

Data were plotted to fit a 4-parameter sigmoid curve in GraphPad Prism software using serum dilution (log transformed) as the x axis variable and AFU on the y axis. Over many iterations during development of this assay, we have determined that sera from naïve volunteers in the United States and Europe, including pre-immune sera, always register an arbitrary fluorescence value less than  $2.0 \times 10^5$  even at the highest concentration (1:50 dilution) used in this assay. Moreover, sera that do react to PfSPZ,  $2.0 \times 10^5$  AFU, fall in the exponential portion of their sigmoidal curves. Therefore,  $2.0 \times 10^5$  has been chosen as a threshold in the aFA assay, and the results for each volunteer for antibodies to PfSPZ are reported as the reciprocal serum dilution at which fluorescence intensity was equal to  $2.0 \times 10^5$  AFU. For this study, the AFU values were calculated for sera collected before immunization and after immunization because individuals pre-exposed to malaria in EG were expected to harbor antibodies to PfSPZs before immunization with PfSPZ Vaccine. Sera were considered positive for seroconversion if their net AFU  $2 \times 10^5$  and AFU  $2 \times 10^5$  ratio, calculated respectively by subtracting the pre-vaccination from the post-vaccination AFU  $2 \times 10^5$  and dividing the post-vaccination by the pre-vaccination AFU  $2 \times 10^5$  were  $\geq 150$  and  $\geq 3.0$ , respectively.

**Functional antibodies to PfSPZ by inhibition of sporozoite invasion (ISI) assay.** HC-04 (1F9) cells (hepatocytes) were cultured in complete medium (10% FBS in DMEM/F12 with 100 units/mL penicillin and 100 µg/mL streptomycin; Gibco by Life Technologies, Grand Island, NY) in (Entactin-Collagen IV-Laminin) ECL-coated 96-well clear bottom black well plates

(Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of  $2.5 \times 10^4$  cells per well, and incubated for 24 hours at 37°C, 5% CO<sub>2</sub> with 85% relative humidity. Twenty-four hours later, cells were infected with  $10^4$  aseptic, purified, cryopreserved PfSPZ per well, without or with sera diluted in an 11-point dilution series from subjects immunized with PfSPZ Vaccine. The assay control included PfSPZ added with media alone. Sera were assessed at pre-immunization (baseline) and predetermined time points after immunization. PfSPZ that had not invaded the HC-04 cells were removed 3 hours later by washing three times with Dulbecco's phosphate-buffered saline, and the cultures were fixed using 4% paraformaldehyde for 15 minutes at room temperature. Differential immunostaining was performed to distinguish between PfSPZ inside the hepatocytes versus PfSPZ outside the hepatocytes. PfSPZ outside the hepatocytes were stained with an anti-PfCSP mAb (2A10, 6.86 µg/mL) (Protein Potential LLC, with permission from New York University School of Medicine) conjugated with Alexa Fluor 633 (far-red) (1 µg/mL; custom-conjugated at GenScript® USA Inc., Piscataway, NJ), a 1:500 dilution. The hepatocytes were then permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature, and the PfSPZ inside the hepatocytes were stained with the anti-PfCSP mAb (2A10, 6.86 µg/mL) conjugated with Alexa Fluor 488 (green; 1 µg/mL, conjugated from GenScript USA Inc., Piscataway, NJ), a 1:500 dilution.

The numbers and intensity of infected hepatocytes (green only) were counted by scanning the plates using an Acumen eX3 laser scanning imaging cytometer. Inhibitory activity at each serum dilution sera was calculated using the following formula:

$$\text{Percent inhibition} = 100 - \left( \frac{\text{Fluorescence values of invaded PfSPZ in presence of serum} \times 100}{\text{Fluorescence values of invaded PfSPZ in media control}} \right)$$

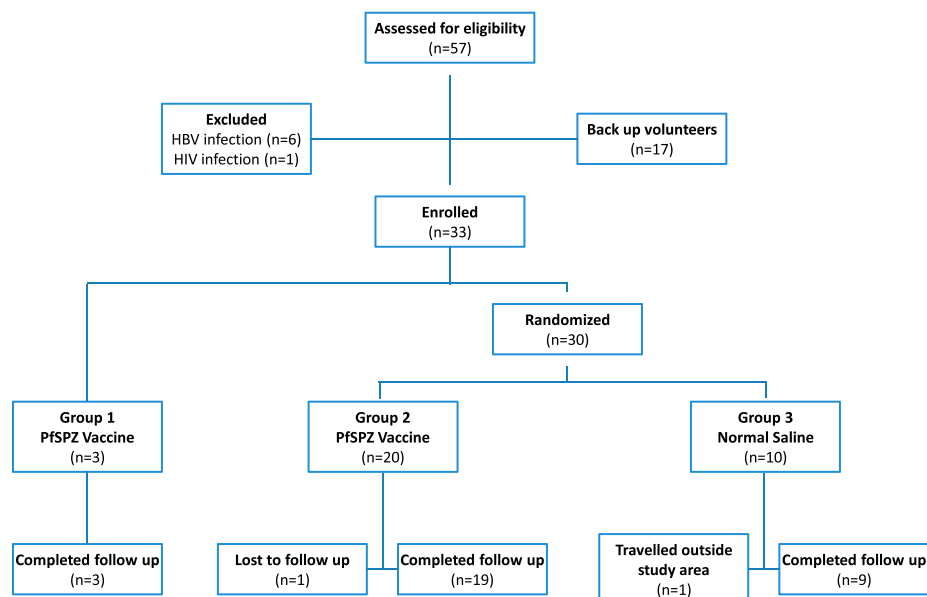


FIGURE 1. Consort diagram for EGSPZV1 clinical trial.

TABLE 1  
Demographic and baseline characteristics by group of study population

	Group 1 PfSPZ Vaccine	Group 2 PfSPZ Vaccine	Group 3 normal saline
Number of volunteers	3	20	10
Mean age (range)	19.7 (19–20)	20.0 (17–26)	22.9 (18–33)
Mean body mass index (range)	22.5 (20.6–24.8)	23.0 (18.8–28.6)	22.1 (19.5–24.0)

PfSPZ = *Plasmodium falciparum* sporozoites.

The number of invaded PfSPZ scored in this assay in the absence of serum was 400–600, giving an intensity of  $1\text{--}3 \times 10^6$  fluorescence units. Data were plotted to fit a 4-parameter sigmoid curve in GraphPad Prism software using serum dilution (log transformed) as the *x* axis variable and percent inhibition on the *y* axis. Eighty percent inhibition was interpolated from the sigmoidal curves as the reciprocal serum dilution at which the fluorescence intensity of infected wells with serum was 20% of the negative control without serum. The serum dilution at which inhibition of PfSPZ invasion into hepatocytes was 80% as compared with a negative control without serum was designated as ISI activity. Sera with a net ISI activity of  $\geq 10\%$  between post- and pre-immunization sera and a ratio of post- to pre-vaccination ISI activity of  $\geq 3.0$  were considered positive.

**Statistical analysis.** This was a phase 1 clinical trial with no formal sample size calculation. We compared the proportion

of subjects with AEs as well as frequencies of individual AEs between vaccination groups recorded from the first vaccination through study conclusion (24 weeks post last vaccination).

The data for local and systemic AEs were grouped by vaccination groups and summarized by frequency and severity of AEs using descriptive statistics. A two-sided Fisher's exact test was used to compare the frequency of AEs between vaccinees and controls. For the immunogenicity analysis, change from the baseline of anti-PfCSP antibodies was plotted by vaccine group and time points together with the median and interquartile ranges. Nonparametric statistics (Wilcoxon-Mann-Whitney test) were used to compare the change of PfSPZ antibody from the baseline between PfSPZ Vaccine and NS groups. The Spearman correlation coefficient was used to assess the association between the results of the different antibody assays. Fixed effect regression model was used to

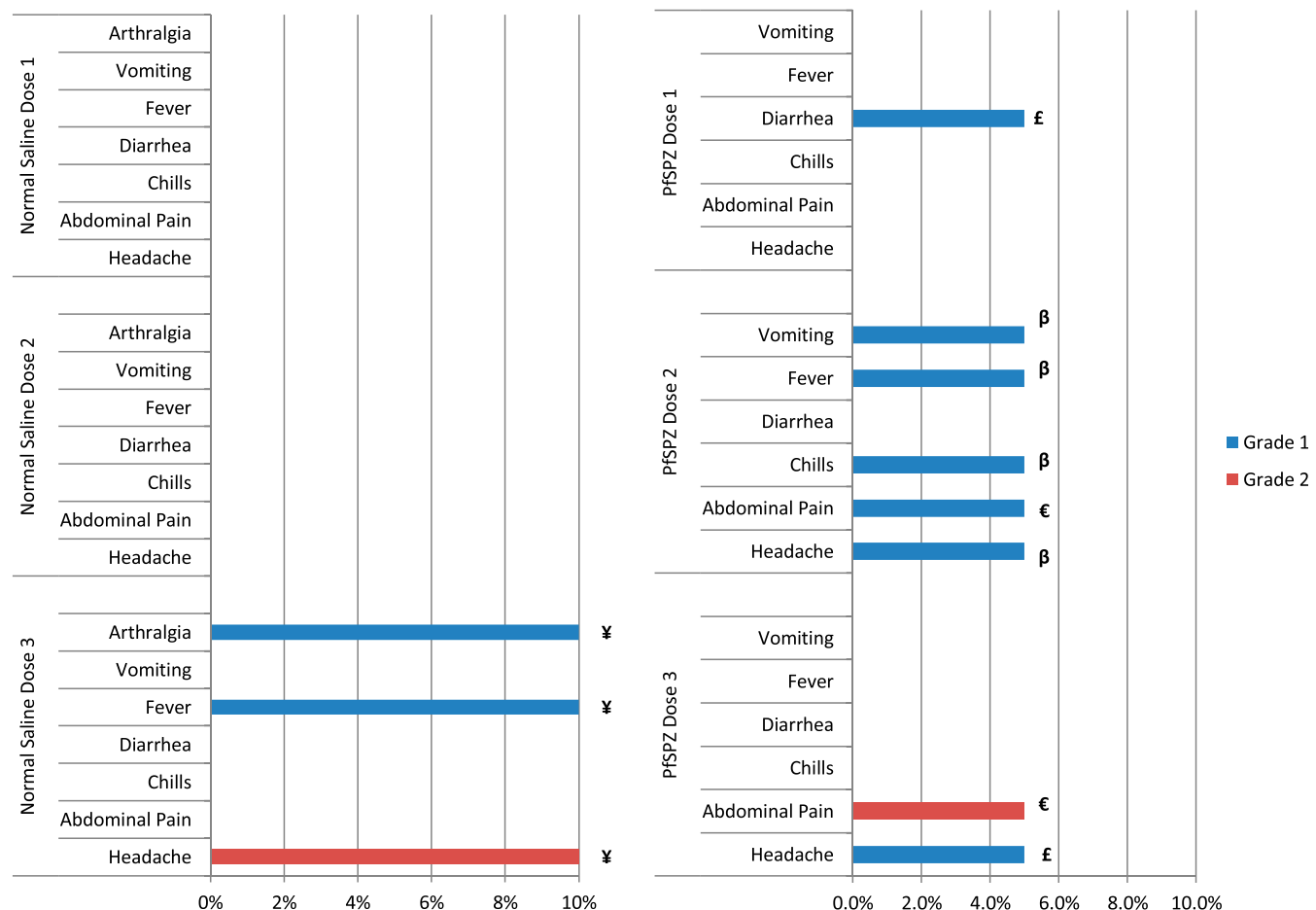


FIGURE 2. Comparison of adverse events (AEs) in volunteers receiving normal saline (NS) and PfSPZ Vaccine. The percent of volunteers with a specific AE is depicted. Only a single volunteer had any individual AE after each immunization (maximum of 1/10 [10%] for NS and 1/20 [5%] for PfSPZ Vaccine). ¥, €, £, β are individual volunteers. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).



evaluate the effect of PfSPZ Vaccine on hematology (white blood cells [WBCs], neutrophils, and eosinophils) parameters taking into account the repeated measurements in each volunteer over time. Data were analyzed using STATA 13.0. Anti-PfCSP antibody activities were compared between different clinical trials using a Kruskal–Wallis test followed by a Dunn's multiple comparisons test for significance between pairs. These data were analyzed using GraphPad Prism 7.

**Genotyping of parasites.** One volunteer developed asymptomatic Pf infection 5 months after the third dose of PfSPZ Vaccine. The Pf parasites from this volunteer were assessed by microsatellite analysis<sup>15</sup> to determine if the parasites were derived from PfSPZ Vaccine (PfNF54) or from a naturally acquired infection. The DNA was isolated from a blood specimen using Quick-gDNA Blood MiniPrep Kit (Zymo Research, Irvine, CA). Unlinked microsatellite markers Poly alpha, PfPK2, TA81, ARA2, TA87, and TA40 were amplified using heminested PCR.<sup>15</sup> Capillary electrophoresis was performed using an Applied Biosystems 3730XL 96-capillary DNA sequencer and software. Capillary electrophoresis output files were analyzed using Geneious 8.1.8 software. The detailed protocol is available on the University of Maryland website (<http://www.medschool.umaryland.edu/malaria/Protocols/>). Genomic control strains NF54, 3D7, 7G8, HB3, and V1S (ATCC-MR4, Manassas, VA) were included to determine characteristic peak morphology for each microsatellite locus and control for slight variations among runs. Peak sizes were determined by manual inspection of each electropherogram and then normalized against the Genescan™ 600 Liz® size standard (Applied Biosystems, Foster City, CA). Normalized peak sizes were compared with those observed for PfNF54.

## RESULTS

**Study population.** Between March and April 2015, 57 adults were screened and of the 40 who met the eligibility

criteria, 33 were enrolled into the study (Figure 1). Three volunteers were enrolled into Group 1, and 30 volunteers were randomized into Groups 2 ( $N = 20$ ) and 3 ( $N = 10$ ). The most common reasons for exclusion were positive HBSAg and/or positive HIV tests. Of 57 volunteers screened, helminth infections were diagnosed in 31 (54%). The mean age at enrollment was 20 (17–26) years in the PfSPZ Vaccine group and 23 (18–33) years in the control group. All the groups were generally similar at enrollment with regard to age and body mass index (Table 1). Of the 33 volunteers, all received all scheduled immunizations, and 31 (94%) completed all follow-up visits. Two volunteers failed to complete their follow-up; one vaccinee traveled abroad for further studies 2 months after the last vaccination, and one control was lost to follow up at 5 months after the last vaccination. The two volunteers were healthy at their last scheduled visits.

**Vaccine administration.** Vaccinations took place over a 4-month period from March through August 2015. Study nurses administered the vaccine by DVI. Injection by DVI was completed in one attempt in 88 of 96 (92%) administrations. The other eight administrations were completed in two attempts. The nurse performing the injection judged the procedure as simple in 87 of the 88 (99%) single-attempt injections. Of 96 administrations, 91 (95%) were judged to be painless by the volunteers, and five (5%) were judged to be associated with mild pain. The entire DVI procedure from the time of syringe handover to completion of injection took a mean of 3.80 (1–8) minutes, 2.19 (1–5) minutes, and 2.40 (1–5) minutes in the safety sentinel, PfSPZ Vaccine, and NS groups, respectively.

**Solicited and unsolicited AEs.** No volunteer developed local or systemic solicited AEs in the sentinel group of three volunteers who received two vaccinations with PfSPZ Vaccine. No local solicited AEs were reported after administration of any of the three doses of PfSPZ Vaccine and NS in the main groups. Overall, three of 20 (15%) and one of 10 (10%) volunteers reported a systemic solicited AE after any of the three doses in the PfSPZ Vaccine and NS groups, respectively (Figure 2). Of the 11 reported systemic AEs, nine (82%) were grade 1, and 2 (18%) were grade 2 (one in the PfSPZ Vaccine and one in the NS group) (Figure 2). All solicited AEs resolved without sequelae within 7 days after vaccination. There was no

TABLE 2  
Number of solicited AEs after an injection in recipients of NS and PfSPZ Vaccine

AE	NS N (%)*	PfSPZ Vaccine N (%)*
Local (site of injection)		
Any AE	0	0
Systemic		
Headache	1 (3%)	2 (3%)
Abdominal pain	0	2 (3%)
Chills	0	1 (2%)
Diarrhea	0	1 (2%)
Vomiting	0	1 (2%)
Arthralgia	1 (3%)	0
Fever	1 (3%)	1 (2%)
Myalgia	0	0
Allergic reactions	0	0
Chest pain/palpitations/shortness of breath	0	0
TOTAL	3 (10%)	8 (13%)

AE = adverse event; N = number of AEs; NS = normal saline; PfSPZ = *Plasmodium falciparum* sporozoites.

% = Percentage of injections which gave rise to the AE using number of injections (60 injections for vaccine group, 30 injections for NS group) as denominator. There were no significant differences in the incidence of solicited AEs between recipients of NS and PfSPZ Vaccine.

\* The  $P$  value for the Fisher exact test (2-sided) comparing the two groups for each AE and overall AE rate was  $> 0.05$  for all comparisons.

TABLE 3  
Number of unsolicited AEs in recipients of NS and PfSPZ Vaccine

Unsolicited AEs (MedRA term)	NS N (%)*	PfSPZ Vaccine N (%)*
Dental and periodontal infections	1 (6.3%)	3 (18.8%)
Ear and labyrinth disorders	0 (0.0%)	1 (6.3%)
Gastrointestinal disorders	0 (0.0%)	1 (6.3%)
General disorders	3 (18.8%)	1 (6.3%)
Infections and infestations	6 (37.5%)	8 (50.0%)
Injury and procedural complications	2 (12.5%)	0 (0.0%)
Musculoskeletal and connective tissue disorders	3 (18.8%)	1 (6.3%)
Nervous system disorders	0 (0.0%)	1 (6.3%)
Skin and subcutaneous tissue disorders	1 (6.3%)	0 (0.0%)
Total number of unsolicited AEs	16	16

AE = adverse event; NS = normal saline; PfSPZ = *Plasmodium falciparum* sporozoites. There were no significant differences in the incidence of unsolicited AEs between recipients of NS and PfSPZ Vaccine. The  $P$  values were calculated on a per volunteer basis (e.g. 3/20 vs. 1/10 for dental and periodontal infections) in a two-sided Fisher's exact test. All  $P$  values were  $\geq 0.1$ .

\* The numbers in brackets are calculated as the proportion of the total unsolicited AEs reported.

evidence of increasing frequency or severity of reported local and/or systemic solicited AEs with subsequent doses of vaccine nor were there reports of anaphylaxis or any other serious allergic reactions. There was no significant difference in the frequency of solicited AEs between PfSPZ Vaccine and NS groups (Table 2).

There were 32 unsolicited AEs reported during the study, of which 27 (84.4%) were mild (grade 1), three (9.4%) were moderate (grade 2), and two (6.2%) were of severe (grade 3) severity (Table 3). The two severe unsolicited AEs were periodontitis and toothache. One volunteer completed treatment of periodontitis and recovered within 16 days. The other volunteer's severe toothache resolved after 2 days. Subsequent evaluation revealed that he had pre-existing dental caries. None of the unsolicited AEs were considered related to the Investigational Product. All other unsolicited AEs resolved without sequelae. There was no statistically significant difference in frequency of unsolicited AEs between volunteers receiving PfSPZ Vaccine and those receiving NS ( $P = 0.319$ , Fisher's exact test, 2-tailed) (Table 3).

One volunteer developed asymptomatic Pf infection 5 months after the third dose of PfSPZ Vaccine. The volunteer was treated with artesunate–amodiaquine, and a repeat blood smear 7 days later confirmed clearance of parasitemia. The microsatellite patterns of the DNA from the parasites from this individual indicated that the volunteer was not infected with PfNF54, the Pf strain used to make PfSPZ Vaccine. The microsatellite analysis indicated that the volunteer was infected with multiple other strains of Pf.

Overall, no unexpected or SAEs occurred. Furthermore, no volunteers were withdrawn because of AEs.

**Laboratory safety tests.** Most of the abnormal hematology (WBC and differentials) and biochemistry (serum creatinine, ALT and AST) laboratory abnormalities were grade 1 and 2 (Table 4). Eosinophilia was a common laboratory abnormality, occurring at some point in most volunteers and often associated with the presence of helminth infections, which included *Ascaris lumbricoides*, *Trichuris trichiura*, and *Schistosoma haematobium*. The only grade 3 laboratory

abnormality identified after vaccination in the main study groups, an elevated eosinophil count in a PfSPZ Vaccine recipient, was associated with infection with *S. haematobium*, and fell to grade 1 after treatment. Transient, grade 1–2 decreases in neutrophil counts were also common, but showed no relationship to administration of PfSPZ Vaccine, occurring more frequently in control volunteers than vaccine recipients. Eosinophils and WBCs had a minimal, but significant downward trend during the study period in the PfSPZ Vaccine group, but not in NS controls (data not shown). Elevated ALT and AST levels, which could theoretically be expected after the administration of a live PfSPZ vaccine, occurred with similar frequency in vaccine and control volunteers. In summary, there were no significant trends or group differences in laboratory parameters indicating a vaccine effect.

**Immunogenicity.** In the PfCSP ELISA, 14/20 (70%) of vaccinated subjects and 0/10 (0%) of control subjects were considered to have become positive as compared with before immunization ( $P = 0.0003$ , Fisher's exact test, 2-tailed) with a median net OD 1.0 of positives of 890 and the median OD 1.0 ratio of positives of 9.50 (Table 5). Among positives, the net OD 1.0 ranged from 136 to 2,414, and the OD 1.0 ratio ranged from 3.10 to 23.66 (Figure 3A) (Table 5).

In the aIFA, 13/20 (65%) of vaccinated subjects and 3/10 (30%) of control subjects were considered to have become positive as compared with before immunization (Figure 3B) (Table 5) ( $P = 0.12$ ). Among positive vaccinees, the net AFU  $2 \times 10^5$  ranged from 150 to 647, and the AFU  $2 \times 10^5$  ratio ranged from 3.10 to 270 (Table 5). Of the 14 vaccinees who seroconverted by PfCSP ELISA, only one did not seroconvert by PfSPZ aIFA (Table 5).

In the ISI, 9/20 vaccinees and 1/10 control subjects were considered to have become positive as compared with before immunization (Figure 3C) (Table 5) ( $P = 0.10$ ). All nine were also positive by PfCSP ELISA, and 8/9 were positive by the aIFA. Among positives, the net ISI activity ranged from 11.42 to 63.23 and the ISI activity ratio ranged from 3.14 to 25.63 (Table 5).

TABLE 4  
Laboratory abnormalities

Laboratory parameter*	Grade abnormality	Pilot N (%)	PfSPZ Vaccine N (%)	Normal saline N (%)
Decreased hemoglobin	1	0 (0%)	0 (0%)	1 (10%)
Decreased platelets	1	0 (0%)	4 (20%)	0 (0%)
Increased WBC count	1	0 (0%)	5 (25%)	2 (20%)
	2	—	1 (5%)	—
Decreased WBC count	1	1 (33%)	5 (25%)	5 (50%)
Decreased neutrophils	1	1 (33%)	17 (85%)	8 (80%)
	2	—	1 (5%)	2 (20%)
Decreased lymphocytes	1	1 (33%)	0 (0%)	1 (10%)
Increased eosinophils	1	0 (0%)	14 (70%)	10 (100%)
	2	—	11 (55%)	3 (30%)
	3	—	1 (5%)	—
Elevated ALT	1	1 (33%)	8 (40%)	4 (40%)
Elevated AST	1	0 (0%)	6 (30%)	3 (30%)
	2	—	2 (10%)	—
Elevated total bilirubin	1	0 (0%)	0 (0%)	0 (0%)
Elevated creatinine	1	1 (33%)	0 (0%)	2 (20%)
Hypoglycemia	1	0 (0%)	2 (10%)	0 (0%)
Hyperglycemia	1	1 (33%)	2 (10%)	1 (10%)
	2	1 (33%)	—	1 (10%)

ALT = alanine aminotransferase; AST = aspartate aminotransferase; N = number of volunteers experiencing the abnormality; PfSPZ = *Plasmodium falciparum* sporozoites; WBC = white blood cell; % = proportion of the number of volunteers in the group experiencing the abnormality. Number (percent) of volunteers experiencing laboratory abnormalities after vaccination (excluding abnormalities identified at screening or prior to the first vaccination).

\* Abnormal Laboratory Values are defined as grade 1 or higher per the protocol defined toxicity ranges.

TABLE 5

Immune responses in volunteers receiving NS and PfSPZ Vaccine before the first immunization and 2 weeks after the third immunization

Group	Volunteer ID	ELISA PfCSP OD 1.0				aIFA AFU $2 \times 10^5$				ISI reciprocal serum dilution for 80% inhibition			
		Pre-immune*	Immune†	Net‡	Ratio§	Pre-immune*	Immune†	Net‡	Ratio§	Pre-immune*	Immune†	Net‡	Ratio§
NS	201	188	342	154	1.82	0	73	73	73.00	<b>4.91</b>	<b>21.33</b>	<b>16.41</b>	<b>4.34</b>
	222	192	278	86	1.45	0	71	71	71.00	15.31	16.31	0.99	1.06
	225	38	37	-1	0.97	<b>73</b>	<b>345</b>	<b>272</b>	<b>4.73</b>	13.72	2.60	-11.13	0.19
	232	102	158	56	1.55	54	81	27	1.50	0.00	8.83	8.83	8.83
	235	62	158	96	2.55	127	216	89	1.70	13.76	14.11	0.36	1.03
	241	167	133	-34	0.80	0	39	39	39.00	9.36	12.88	3.52	1.38
	251	654	481	-173	0.74	138	0	-138	0.00	17.43	3.75	-13.69	0.21
	253	45	47	2	1.04	19	104	85	5.47	10.17	21.02	10.84	2.07
	256	95	210	115	2.21	<b>0</b>	<b>238</b>	<b>238</b>	<b>238.00</b>	13.15	25.61	12.46	1.95
	258	39	40	1	1.03	<b>0</b>	<b>372</b>	<b>372</b>	<b>372.00</b>	7.31	7.55	0.23	1.03
PfSPZ Vaccine	Median	99	158	29	1.25	19	93	79	22.24	11.66	13.50	2.26	1.22
	203	<b>46</b>	<b>808</b>	<b>762</b>	<b>17.57</b>	<b>0</b>	<b>270</b>	<b>270</b>	<b>270.00</b>	<b>0.00</b>	<b>9.93</b>	<b>9.93</b>	<b>9.93</b>
	204	328	551	223	1.68	134	122	-12	0.91	12.51	28.69	16.18	2.29
	206	275	412	137	1.50	212	268	56	1.26	16.05	5.75	-10.29	0.36
	212	<b>172</b>	<b>1,472</b>	<b>1,300</b>	<b>8.56</b>	<b>18</b>	<b>252</b>	<b>234</b>	<b>14.00</b>	<b>8.60</b>	<b>67.53</b>	<b>58.93</b>	<b>7.85</b>
	215	<b>77</b>	<b>603</b>	<b>526</b>	<b>7.83</b>	<b>88</b>	<b>488</b>	<b>400</b>	<b>5.55</b>	<b>0.00</b>	<b>14.06</b>	<b>14.06</b>	<b>14.06</b>
	217	<b>85</b>	<b>938</b>	<b>853</b>	<b>11.04</b>	<b>74</b>	<b>470</b>	<b>396</b>	<b>6.35</b>	<b>4.55</b>	<b>20.71</b>	<b>16.15</b>	<b>4.55</b>
	218	<b>89</b>	<b>736</b>	<b>647</b>	<b>8.27</b>	<b>82</b>	<b>254</b>	<b>172</b>	<b>3.10</b>	13.55	31.99	18.44	2.36
	226	311	381	70	1.23	259	340	81	1.31	5.65	2.44	-3.22	0.43
	227	525	536	11	1.02	417	293	-124	0.70	12.67	27.71	15.04	2.19
	229	<b>237</b>	<b>1,314</b>	<b>1,077</b>	<b>5.54</b>	<b>127</b>	<b>538</b>	<b>411</b>	<b>4.24</b>	21.80	33.79	11.99	1.55
	231	<b>118</b>	<b>1,547</b>	<b>1,429</b>	<b>13.11</b>	<b>84</b>	<b>404</b>	<b>320</b>	<b>4.81</b>	<b>0.00</b>	<b>39.64</b>	<b>39.64</b>	<b>39.64</b>
	233	<b>199</b>	<b>1,472</b>	<b>1,273</b>	<b>7.40</b>	<b>59</b>	<b>706</b>	<b>647</b>	<b>11.97</b>	<b>0.00</b>	<b>20.32</b>	<b>20.32</b>	<b>20.32</b>
	236	<b>62</b>	<b>198</b>	<b>136</b>	<b>3.19</b>	299	292	-7	0.98	<b>3.07</b>	<b>20.03</b>	<b>16.96</b>	<b>6.52</b>
	242	<b>47</b>	<b>755</b>	<b>708</b>	<b>16.06</b>	<b>16</b>	<b>341</b>	<b>325</b>	<b>21.31</b>	21.38	39.39	18.01	1.84
	243	<b>188</b>	<b>1,115</b>	<b>927</b>	<b>5.93</b>	<b>0</b>	<b>329</b>	<b>329</b>	<b>329.00</b>	15.27	5.53	-9.74	0.36
	244	<b>23</b>	<b>240</b>	<b>217</b>	<b>10.43</b>	<b>0</b>	<b>151</b>	<b>151</b>	<b>151.00</b>	<b>0.00</b>	<b>11.18</b>	<b>11.18</b>	<b>11.18</b>
	249	106	219	113	2.07	71	147	76	2.07	10.95	18.46	7.51	1.69
	250	<b>47</b>	<b>1,112</b>	<b>1,065</b>	<b>23.66</b>	<b>3</b>	<b>512</b>	<b>509</b>	<b>170.67</b>	21.35	46.07	24.72	2.16
	252	<b>236</b>	<b>2,650</b>	<b>2,414</b>	<b>11.23</b>	<b>44</b>	<b>272</b>	<b>228</b>	<b>6.18</b>	<b>8.13</b>	<b>27.76</b>	<b>19.63</b>	<b>3.41</b>
	257	42	90	48	2.14	45	107	62	2.38	22.56	19.12	-3.43	0.85
	Median	112	746	678	7.61	72	292	231	5.18	9.77	20.51	15.60	2.33

AFU = arbitrary fluorescence units; aIFA = automated immunofluorescence assay; ELISA = enzyme-linked immunosorbent assay; ISI = inhibition of sporozoite invasion assay; NS = normal saline; PfCSP = *Plasmodium falciparum* circumsporozoite protein; PfSPZ = *P. falciparum* sporozoites; Volunteer ID = volunteer identification number. Immune responses were measured in all Group 2 and Group 3 volunteers using PfCSP ELISA, aIFA, and ISI assays. Those in bold were considered to have developed antibodies after immunization.

\* Pre-immune sera taken before the first immunization.

† Immune sera taken 2 weeks after the last immunization.

‡ Net = immune-pre-immune value.

§ Ratio = immune/pre-immune value. All values of 0 were changed to 1 when calculating ratios.

The antibody responses by ELISA (net OD 1.0) and net ISI activity (80% inhibition of PfSPZ invasion) were positively correlated 2 weeks after the third dose ( $r^2 = 0.39$ ,  $P = 0.0033$ ); the same was true for correlation between PfCSP ELISA and net aIFA ( $r^2 = 0.52$ ,  $P = 0.0003$ ). There was no significant correlation between net aIFA and net ISI ( $r^2 = 0.11$ ,  $P = 0.16$ ).

We compared the anti-PfCSP responses by ELISA at 2 weeks (United States, Mali, EG) or 4 weeks (Tanzania) after administration of three doses at 8-week intervals of  $2.7 \times 10^5$  PfSPZ to adults in EG, to responses to three doses of  $2.7 \times 10^5$  PfSPZ administered to adults at 4-week intervals in Mali<sup>7</sup> and Tanzania (Jongo, unpublished data), and in the United States at 4-week intervals or over 20 weeks<sup>8,9</sup> (Figure 4). Responses were significantly lower in Mali and EG than in the United States, and the responses in EG were significantly lower than in Tanzanian young adults who had minimal exposure to malaria during the previous 5 years. There was no difference in responses in sera from Mali and EG.

## DISCUSSION

We have conducted the first clinical trial in the history of EG. There were significant regulatory, quality, clinical, and

logistical challenges in conducting this trial fully adhering to the international standards of Good Clinical Practices. A detailed report of these developments and capacity building efforts will be published elsewhere. The trial was successful in showing that three doses of  $2.7 \times 10^5$  PfSPZ of PfSPZ Vaccine were safe, well tolerated, and moderately immunogenic in healthy 18–35-year-old male Equatoguineans living in an area with low malaria exposure.

Almost all the vaccinees and controls indicated that there was no pain associated with injection of PfSPZ Vaccine by DVI. There were no differences in the rate of solicited or unsolicited AEs between the 20 volunteers who received PfSPZ Vaccine and the 10 who received NS. There were no local solicited AEs or severe solicited systemic AEs after vaccination, and only two grade 3 unsolicited AEs were reported during the trial, both unrelated to investigational product. We observed no unexpected trends in hematological (hemoglobin, hematocrit, WBCs, platelets) or biochemical (creatinine, ALT, AST) laboratory markers except that eosinophils and WBCs had a minimal, but significant downward trend during the study period in the PfSPZ Vaccine group, but not in NS controls. We have no explanation for this finding. The excellent reactivity and safety profile in Equatoguineans immunized with PfSPZ Vaccine is consistent with findings from Mali<sup>7</sup> and Tanzania

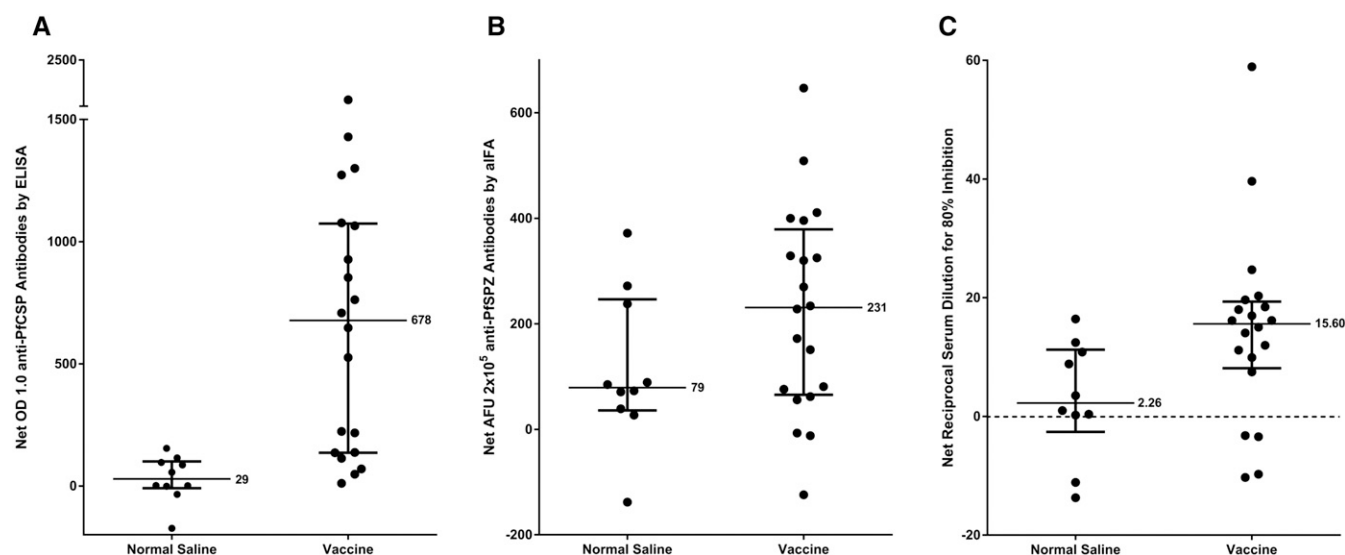


FIGURE 3. Antibody responses 2 weeks after the last vaccination. Responses in all vaccinees and controls who received three doses of PfSPZ Vaccine or normal saline were measured by (A) *Plasmodium falciparum* (Pf) circumsporozoite protein (PfCSP) enzyme-linked immunosorbent assay (ELISA), (B) Automated immunofluorescence assay (alFA), and (C) Inhibition of sporozoite invasion assay (ISI) assay. In the ELISA, antibody responses are reported as the net OD 1.0. OD 1.0 is the serum dilution at which the optical density is 1.0, and net OD 1.0 is the difference between the post- and pre-immunization OD 1.0. In the alFA assay, antibody responses are reported as the reciprocal serum dilution at which the arbitrary fluorescence units (AFU) were  $2 \times 10^5$ . In the ISI assay, antibody responses are reported as the reciprocal serum dilution that gave 80% inhibition of PfSPZ invasion. Medians with interquartile ranges are shown.

(Jongo et al., unpublished data) where similar doses of PfSPZ Vaccine were administered. Higher doses of PfSPZ Vaccine are currently being assessed in infants, children, and adults in EG.

The tolerability and safety profiles are also consistent with results of multiple other studies of PfSPZ Vaccine,<sup>6–10,13</sup> PfSPZ Challenge (live PfSPZ for infection),<sup>16–19</sup> and PfSPZ-CVac (PfSPZ Challenge with an antimalarial drug).<sup>20,21</sup> So far, no breakthrough malaria infections have been observed with Sanaria's radiation-attenuated PfSPZ Vaccine. There was no evidence for local or severe systemic AEs, thought to be due to the quality (e.g., purity and sterility) of PfSPZ Vaccine. This favorable safety and tolerability profile will facilitate compliance if PfSPZ Vaccine is used, as planned,<sup>22,23</sup> in mass vaccination programs.

PfSPZ Vaccine induced antibody responses to PfCSP and PfSPZ. However, induction of functional activity assessed by ISI was modest. The antibody responses in this EG study were significantly lower than in a U.S. study in which the same immunization regimen was administered, but similar to antibody responses in a study in a highly endemic area of Mali (Figure 4). Interestingly, antibody responses were higher in young adult Tanzanians who were selected for having had minimal exposure to malaria during the past 5 years (Figure 4). We hypothesize that long-term exposure to Pf infections leads to downregulation of immune responses to PfSPZ Vaccine. It is also possible that an activated immune microenvironment, compared with that in Americans, reduced the antibody responses. This has been shown for a yellow fever vaccine study in Entebbe, Uganda.<sup>24</sup> Additional studies will be needed to better explain and overcome this poor immunogenicity.

The baseline rate of helminth infection (54%) was high. The most common helminths were *A. lumbricoides*, *T. trichiura* and *S. haematobium*. Helminth infection can impair responses

to vaccines against various diseases,<sup>25–27</sup> and specifically impaired antibody responses after vaccination with subunit malaria vaccines in humans<sup>28</sup> and mice.<sup>29,30</sup> In contrast, helminth infection did not affect the immunogenicity and protection of radiation-attenuated SPZ vaccine in mice.<sup>31</sup> The helminth rate at enrollment was significantly lower in Mali (3/93 (3.2%))<sup>7</sup> where antibody responses were similarly low (Figure 4). Thus, we do not think that active, ongoing helminth infections alone were responsible for the poor antibody responses. Nonetheless, we will continue to study the impact of helminth infections on immunogenicity and protective efficacy where possible.

Trained study nurses performed all of the DVI injections of PfSPZ Vaccine and NS. The procedure took little time to learn or to perform and was largely painless. At the time of writing this manuscript, > 4,000 injections by DVI of PfSPZ Vaccine, PfSPZ-CVac,<sup>20,21</sup> PfSPZ Challenge,<sup>16–19</sup> or NS placebo have been administered to > 1,900 individuals worldwide, including infants and children in Tanzania, Kenya, and EG. It is more challenging to administer by DVI to children less than 2 years of age than to older children, adolescents, and adults. However, experience from studies conducted in Mali and Tanzania shows that injection skills improve significantly with practice, and more than 350 5–12-month-old infants have successfully received PfSPZ Vaccine or NS placebo by DVI. Data from an ongoing PfSPZ Vaccine study in Kenya including more than 300 infants will provide even more insight into the operational requirements for DVI in young children and infants.

In conclusion, PfSPZ Vaccine was safe and well tolerated in young Equatoguinean adults living in an area of low malaria transmission, but the vaccine induced lower anti-PfCSP antibody responses compared with those in malaria-naïve adults. Further studies are ongoing to improve the immunogenicity through dose escalation. By successfully conducting the first clinical trial in the country's history, we have laid the foundation for development of a robust research and

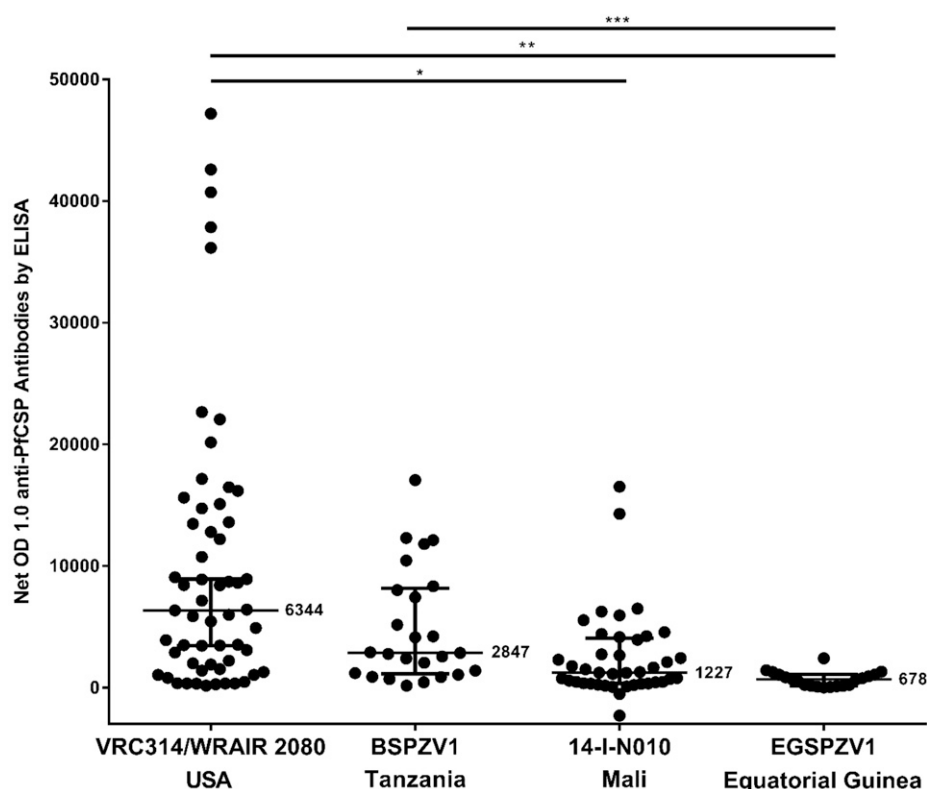


FIGURE 4. Comparison of anti-*Plasmodium falciparum* circumsporozoite protein (PfCSP) antibody responses in four different clinical trials in which the same dose of PfSPZ Vaccine was administered. Antibody responses measured by enzyme-linked immunosorbent assay (ELISA) are reported as the net OD 1.0. The OD 1.0 is the serum dilution at which the optical density is 1.0, and net OD 1.0 is the difference between the post- and pre-immunization OD 1.0. In the USA (VRC314/WRAIR 2080) (most subjects), Mali and Tanzania vaccinations were at 0, 4, and 8 weeks. In Equatorial Guinea (EG), vaccinations were at 0, 8, and 16 weeks. In the United States, Mali, and EG, sera were drawn at 2 weeks after the last vaccination. In Tanzania, sera were drawn at 4 weeks after the last vaccination. Bars with asterisks indicate the statistical significance, as determined by a Kruskal–Wallis test followed by a Dunn’s multiple comparisons test ( $P = * < 0.0001$ ;  $** < 0.0001$ ;  $*** 0.0008$ ).

development program in EG that will contribute to future malaria elimination efforts.

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## Safety and Differential Antibody and T-Cell Responses to the *Plasmodium falciparum* Sporozoite Malaria Vaccine, PfSPZ Vaccine, by Age in Tanzanian Adults, Adolescents, Children, and Infants

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**Abstract.** In 2016, there were more cases and deaths caused by malaria globally than in 2015. An effective vaccine would be an ideal additional tool for reducing malaria's impact. Sanaria® PfSPZ Vaccine, composed of radiation-attenuated, aseptic, purified, cryopreserved *Plasmodium falciparum* (Pf) sporozoites (SPZ) has been well tolerated and safe in malaria-naïve and experienced adults in the United States and Mali and protective against controlled human malaria infection with Pf in the United States and field transmission of Pf in Mali, but had not been assessed in younger age groups. We, therefore, evaluated PfSPZ Vaccine in 93 Tanzanians aged 45 years to 6 months in a randomized, double-blind, normal saline placebo-controlled trial. There were no significant differences in adverse events between vaccinees and controls or between dosage regimens. Because all age groups received three doses of  $9.0 \times 10^5$  PfSPZ of PfSPZ Vaccine, immune responses were compared at this dosage. Median antibody responses against Pf circumsporozoite protein and PfSPZ were highest in infants and lowest in adults. T-cell responses were highest in 6–10-year olds after one dose and 1–5-year olds after three doses; infants had no significant positive T-cell responses. The safety data were used to support initiation of trials in > 300 infants in Kenya and Equatorial Guinea. Because PfSPZ Vaccine-induced protection is thought to be mediated by T cells, the T-cell data suggest PfSPZ Vaccine may be more protective in children than in adults, whereas infants may not be immunologically mature enough to respond to the PfSPZ Vaccine immunization regimen assessed.

### INTRODUCTION

Despite an annual investment of more than \$2.7 billion in insecticide-treated bed nets, indoor residual spraying, diagnosis, and treatment, in 2015, 2016, and 2017 there were an estimated 429,000–730,500 deaths each year caused by malaria<sup>1–3</sup>; 90% of the mortality was in children under the age of 5 years. *Plasmodium falciparum* (Pf) was the cause of more than 98% of deaths from malaria and more than 80% of cases of malaria in sub-Saharan Africa. Our goal is to field a vaccine that will prevent infection with Pf and thereby prevent all clinical and pathological manifestations of malaria and halt parasite transmission from humans to mosquitoes.<sup>4</sup>

A number of malaria vaccines are under development, but none have received marketing authorization (licensing) by a regulatory authority. RTS,S/AS01 has completed Phase 3 clinical trials,<sup>5</sup> received a positive opinion (Article 58) from the European Medicines Agency<sup>6</sup> and in 2019, large-scale pilot implementation trials will be initiated in Kenya, Malawi, and Ghana to confirm the level of protective efficacy, demonstrate that the entire immunization regimen can be successfully administered, and assess several safety signals seen in the Phase 3 trial (increased meningitis, febrile seizures, and female mortality in vaccinees as compared with controls).<sup>7,8</sup> A second pre-erythrocytic stage vaccine ChAd63 and MVA ME-TRAP has also been studied in African infants to adults.<sup>9–11</sup>

Sanaria® PfSPZ Vaccine is composed of radiation-attenuated, aseptic, purified, and cryopreserved *Plasmodium falciparum* (Pf) sporozoites (SPZ).<sup>12</sup> The vaccine has been extremely well tolerated and safe in multiple clinical trials.<sup>13–18</sup> In Mali, Equatorial Guinea, and Tanzania, there was no difference in adverse events (AEs) between the PfSPZ Vaccine and normal saline (NS) control in double-blind, placebo-controlled trials.<sup>18–20</sup>

PfSPZ Vaccine has been reported in malaria-naïve adults to have a vaccine efficacy (VE) of > 90% against controlled human malaria infection (CHMI) with homologous Pf parasites (same Pf strain in vaccine and CHMI),<sup>14,16</sup> 80% against CHMI with heterologous Pf parasites (different Pf strain in vaccine and CHMI) 3 weeks after the last vaccine dose,<sup>14,16</sup> 65% and 55% against homologous CHMI 24,<sup>16</sup> and 59<sup>15</sup> weeks and 54% against heterologous CHMI 33 weeks after the last vaccine dose.<sup>17</sup> In Malian adults, VE against Pf infection during the 24 weeks after last vaccine dose was 52% by time to infection analysis and 29% by proportional analysis.<sup>18</sup> Protection by immunization with sporozoites is dependent on T cells in mice and nonhuman primates<sup>13,21–24</sup> and thought to be T cell-dependent in humans.<sup>13</sup> The durable protection demonstrated in the Mali trial was associated with elevated gamma delta T-cell frequencies, providing support for this hypothesis.<sup>25</sup>

However, in Tanzanian adults, five doses of  $2.7 \times 10^6$  PfSPZ had a VE against 3- and 24-week homologous CHMI of 20%.<sup>20</sup> This was the same immunization regimen used in the Mali trial that gave 52% VE and in a trial in the United States that gave 92% and 65% VE against 3- and 24-week homologous CHMI.<sup>16</sup> In Tanzania, the antibody and T-cell responses to PfSPZ in adults were significantly lower than in adults in the United States<sup>20</sup>; antibody responses in Mali were even

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lower.<sup>18</sup> We hypothesized that the lower immune responses in malaria-exposed African subjects as compared with malaria-naïve U.S. subjects were due to immune dysregulation caused by long-term exposure to malaria parasites<sup>18,20</sup> and that naturally acquired immunity may have reduced the effective PfSPZ inoculum. We, therefore, proposed that injecting larger doses of PfSPZ might partially overcome these effects. This is in part because when humans are immunized with radiation-attenuated PfSPZ administered by mosquito bite,<sup>26</sup> PfSPZ Vaccine<sup>14,15,17,27</sup> and PfSPZ-CVac<sup>27</sup> protection is dose dependent.<sup>26</sup> Thus, increasing immune responses by increasing the dose should increase VE. Thus, in this study, we increased the dose of PfSPZ Vaccine from  $2.7 \times 10^5$  PfSPZ to  $9.0 \times 10^5$  PfSPZ and  $1.8 \times 10^6$  PfSPZ.

All previous studies of PfSPZ Vaccine have been conducted in adults. However, the major burden of malaria is in older infants and children. The present study was the first to assess the tolerability, safety, and immunogenicity of PfSPZ Vaccine in adolescents, children, and infants aged 6 months and older and the first to compare these results with those of adults. Furthermore, we hypothesized that infants and young children with little previous exposure to Pf parasites would have more robust immune responses to the vaccine than adults, recognizing that the infants' immunological systems might not be fully mature, particularly for T-cell responses.<sup>28</sup>

## MATERIAL AND METHODS

**Study design and population.** This single-center, age de-escalation, double-blind, randomized, placebo-controlled trial (ClinicalTrials registration no. NCT02613520) was conducted in Bagamoyo, Tanzania, between December 2015 and March 2017. It had two major components, an age de-escalation, dose escalation component to assess safety, tolerability, and immunogenicity of PfSPZ Vaccine (part A), and a CHMI component to assess VE (part B). Herein, we report the results of part A.

One hundred seventy-three healthy male and female volunteers aged 6 months to 45 years were recruited from the Bagamoyo region through locally presented sensitization meetings. After an initial screening, prospective volunteers were invited to the Bagamoyo Clinical Trial Unit (BCTU) of the Ifakara Health Institute (IHI) to complete the informed consent process and further screening.

Informed consent was obtained from all volunteers or the parents/legal guardians after the nature and risks of the study were explained. Following this, the adult volunteers or the parent/legal guardian of child volunteers were required to complete a 10-question assessment with a 100% correct response rate on the first or second attempt to demonstrate understanding of the study procedures (Supplemental Table 1) to be eligible for enrollment. In addition, all children and adolescents aged 9–18 years provided written assent and children aged 6–8 years provided oral assent. Volunteers were screened using predetermined inclusion and exclusion criteria based on clinical examinations and laboratory tests (Supplemental Tables 2 and 3). Medical history was analyzed to exclude any past or present medical problem in conjunction with a detailed clinical examination. Laboratory testing included hematology, biochemistry, urinalysis, and parasitology testing to include malaria thick blood smear (TBS), stool for intestinal helminth infections, and urine for *Schistosoma*

*haematobium*. Tests for HIV and hepatitis B and C were performed only after pretest counseling was carried out; volunteers were excluded if positive and referred for further evaluation and management. Volunteers were excluded if they had significant abnormalities on electrocardiograms. The complete eligibility criteria are published at <https://clinicaltrials.gov/show/NCT02613520>.

The trial was performed in accordance with good clinical practices. The protocol was approved by institutional review boards (IRBs) of the IHI (Ref. No. IHI/IRB/ No: 32-2015), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/2049), and the Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland (reference number 15/104). The protocol was also approved by the Tanzania Food and Drug Authority (Auth. No. TZ15CT013), registered at ClinicalTrials.gov (NCT02613520) and conducted under a U.S. Food and Drug Administration Investigational New Drug application (FDA IND) application.

**Intervention and randomization.** Volunteers spanning five age groups were sequentially allocated to 11 different dose groups and randomly assigned to receive PfSPZ Vaccine or NS in a 2:1 ratio. Twelve additional, age-matched, adult volunteers were enrolled as nonimmunized infectivity controls for CHMI studies.

The details of each of five main age groups (Groups 1–5) are outlined in Table 1. Immunization began with the adults (Group 1a,  $9.0 \times 10^5$  PfSPZ and Group 1b,  $1.8 \times 10^6$  PfSPZ) and continued progressively to teenagers (11–17 years; Group 2a,  $9.0 \times 10^5$  PfSPZ and Group 2b,  $1.8 \times 10^6$  PfSPZ), older children (6–10 years; Group 3a,  $9.0 \times 10^5$  PfSPZ and Group 3b,  $1.8 \times 10^6$  PfSPZ), younger children (1–5 years; Group 4a,  $4.5 \times 10^5$  PfSPZ and Group 4b,  $9.0 \times 10^5$  PfSPZ), and infants (6–11 months; Group 5a,  $2.7 \times 10^5$  PfSPZ, Group 5b,  $4.5 \times 10^5$  PfSPZ, and Group 5c,  $9.0 \times 10^5$  PfSPZ). Only after the safety of a given PfSPZ dose had been demonstrated in an older age group was the same or a lower dose tested next in a younger age group (age de-escalation). Likewise, within each age group, safety was demonstrated with a lower PfSPZ dose before immunizations began with a higher PfSPZ (dose escalation). At three time points during age de-escalation and dose escalation, a three-member external Safety Monitoring Committee reviewed safety reports and provided a recommendation to proceed to the next study group.

Using this staggered approach, 10 subgroups of nine individuals received three doses of PfSPZ Vaccine at 56-day intervals (days 1, 57, and 113), each comprising six vaccine and three placebo volunteers randomized in a 2:1 ratio to PfSPZ Vaccine or NS control. An exception was Group 5a (Table 1), an 11th subgroup comprising three infants constituting the first ever recipients less than 1 year of age to receive injections with PfSPZ Vaccine. This safety-only pilot group was administered a single reduced dose of PfSPZ Vaccine ( $2.7 \times 10^5$  PfSPZ); there was no randomization and no placebo control. All of the other 90 volunteers and the entire clinical team excluding the pharmacy staff were blinded to treatment assignment, with blinded status maintained throughout the study period.

**Investigational product (IP).** The IP used in this trial, Sanaria PfSPZ Vaccine,<sup>12–19</sup> consists of aseptic, purified, vialled, metabolically active, nonreplicating (live, radiation attenuated) cryopreserved PfSPZ stored in liquid nitrogen vapor phase at  $-150$  to  $-196^\circ\text{C}$ . Preparation of IP was done under the supervision of the study pharmacist, who was not blinded



TABLE 1

Vaccine and control groups by age, vaccine dose, dosing schedule, number of doses, and total number of *Plasmodium falciparum* sporozoites (PfSPZ)

Groups	Age (years)	Subgroups details		N	PfSPZ/dose	Dosing schedule (weeks)	No. doses	Total PfSPZ
Group 1	18–45	1a	Vaccinees	6	$9 \times 10^5$	0, 8, 16	3	$2.7 \times 10^6$
			NS controls	3	NS	0, 8, 16	3	0
		1b	Vaccinees	6	$1.8 \times 10^6$	0, 8, 16	3	$5.4 \times 10^6$
			NS controls	3	NS	0, 8, 16	3	0
Group 2	11–17	2a	Vaccinees	6	$9 \times 10^5$	0, 8, 16	3	$2.7 \times 10^6$
			NS controls	3	NS	0, 8, 16	3	0
		2b	Vaccinees	6	$1.8 \times 10^6$	0, 8, 16	3	$5.4 \times 10^6$
			NS controls	3	NS	0, 8, 16	3	0
Group 3	6–10	3a	Vaccinees	6	$9 \times 10^5$	0, 8, 16	3	$2.7 \times 10^6$
			NS controls	3	NS	0, 8, 16	3	0
		3b	Vaccinees	6	$1.8 \times 10^6$	0, 8, 16	3	$5.4 \times 10^6$
			NS controls	3	NS	0, 8, 16	3	0
Group 4	1–5	4a	Vaccinees	6	$4.5 \times 10^5$	0, 8, 16	3	$1.35 \times 10^6$
			NS controls	3	NS	0, 8, 16	3	0
		4b	Vaccinees	6	$9.0 \times 10^5$	0, 8, 16	3	$2.7 \times 10^6$
			NS controls	3	NS	0, 8, 16	3	0
Group 5	6–11 months	5a	Vaccinees	3	$2.7 \times 10^5$	0	1	$2.7 \times 10^5$
		5b	Vaccinees	6	$4.5 \times 10^5$	0, 8, 16	3	$1.35 \times 10^6$
			NS controls	3	NS	0, 8, 16	3	0
		5c	Vaccinees	6	$9 \times 10^5$	0, 8, 16	3	$2.7 \times 10^6$
			NS controls	3	NS	0, 8, 16	3	0
Total				93				

NS = normal saline.

to the study treatment for each volunteer. Vials of PfSPZ Vaccine were thawed and diluted with phosphate-buffered saline containing human serum albumin and the appropriate numbers of PfSPZ in a final volume of 0.5 mL and drawn into a 1-mL syringe. A volume of 0.5 mL of NS was similarly drawn into a 1-mL syringe for placebo recipients. Dilution and syringe preparation were performed under aseptic conditions in a biological safety cabinet. The pharmacist then handed the appropriate syringe for the specific study subject to the blinded nurse through a window. Reconstituted PfSPZ is a clear, odorless, nonviscous liquid indistinguishable from NS. PfSPZ Vaccine or NS was administered by direct venous inoculation (DVI) through a 25 G  $\times$  16-mm needle. In infants and young children, there was an option to administer the vaccine through a 24-gauge peripheral intravenous catheter.

**Assessment of vaccine safety, tolerability of DVI, and AEs.** After vaccination, volunteers under the age of 18 years were observed at the BCTU for approximately 24 hours during which enquiry of AEs and focused physical examinations were performed at 1, 6, and 12 hours after vaccination and at the time of discharge. Adult volunteers were observed at the BCTU for approximately 2 hours during which enquiry of AEs and focused physical examinations were carried out at 1 hour after vaccination and at the time of discharge. Volunteers were given diaries and thermometers for recording of AEs and temperatures thereafter. Volunteers were seen for safety follow-up visits 2, 7, and 14 days after vaccination, with additional follow-up visits by telephone on days 3–6 (and Day 1 for adults). Children and infants were also evaluated again 28 and 56 days after the final immunization.

Local (site of injection) signs and symptoms were solicited in the 2 days following vaccination for adults, teenagers, and older children (groups 1–3) or 7 days following vaccination for younger children and infants (groups 4 and 5). Systemic signs and symptoms were assessed for 7 days for all groups (Supplemental Table 4). In addition, open-ended questioning

was used to identify unsolicited AEs through day 28 postimmunization.

During the period of follow-up, all solicited and unsolicited events were recorded and graded by the attending physician as follows: mild (no effect on activities), moderate (some interference with normal activity), severe (prevented normal activity and required medical intervention), life-threatening (hospitalization, immediate medical intervention, or therapy required to prevent death), or death. Axillary temperature was recorded as Grade 1 ( $> 37.9$ – $38.4^\circ\text{C}$ ), Grade 2 ( $> 38.4$ – $38.9^\circ\text{C}$ ), Grade 3 ( $> 38.9$ – $40.0^\circ\text{C}$ ), or Grade 4 ( $> 40.0^\circ\text{C}$ ). Hematological and biochemical abnormalities were also assessed at prespecified intervals as defined in the toxicity table of the study protocol, including prevaccination and 7 days postvaccination.

**Malaria parasite diagnosis.** All participants were screened for malaria parasites at baseline and before vaccination by TBS microscopy and retrospectively by quantitative polymerase chain reaction (qPCR). Slide preparation and reading for TBS's were performed according to standard procedures.<sup>29</sup> The theoretical limit of detection of TBS was 2 parasites/ $\mu\text{L}$  (0.5  $\mu\text{L}$  blood examined) for standard reads and 0.5 parasites/ $\mu\text{L}$  (2.0  $\mu\text{L}$  blood examined) for expanded reads done when a volunteer was symptomatic. Quantitative polymerase chain reaction analyses were based on DNA extracted from 180  $\mu\text{L}$  whole blood and amplification of the *pan-Plasmodium* 18S gene<sup>30</sup> and the Pf-specific telomere-associated repetitive element 2<sup>31</sup> following essentially the published procedures. The 18S gene DNA qPCR had a sensitivity of 50 parasites/mL. *Plasmodium malariae* (Pm) cases were identified by a qPCR species identification assay using Pm-specific amplification of plasmepsin 4 as described.<sup>32</sup>

**Genotyping of parasites.** DNA from Pf-positive samples was used to genotype the parasites based on Pf Merozoite Surface Protein-1 (PfMSP-1) and PfMSP-2 gene polymorphisms<sup>33</sup> as well as seven microsatellite markers.<sup>34</sup> All Pf strains were compared with the Pf vaccine strain (NF54).

**Antibody assays.** Sera were assessed for antibodies by enzyme-linked immunosorbent assay (ELISA) to the major protein on the surface of sporozoites (Pf circumsporozoite protein [PfCSP]), immunofluorescence assay (aIFA) to air-dried PfSPZ, and inhibition of sporozoite (PfSPZ) invasion (aISI) of HC-04 cells (hepatocytes) as described.<sup>27</sup>

**T-cell assays.** T-cell responses in cryopreserved peripheral blood mononuclear cells (PBMCs) were assessed by flow cytometry as described.<sup>15</sup>

**Statistical analysis.** The sample sizes of three to six vaccinees in each age group dosage group, 12–15 vaccinees in each age category, and six controls in each age category were selected to be appropriate for the initial assessment of safety, tolerance, and immunogenicity of an investigational vaccine. Categorical variables were summarized using absolute (*n*) and relative (%) frequencies. Continuous variables were summarized using mean and SD, median, and range. Comparisons of categorical variables between groups were analyzed using Barnard's two-sided exact unconditional test, or a two-sided Mantel-Haenszel test stratifying by age group. No corrections were made for multiple comparisons because of the early phase nature of this trial. Analyses of immunological data are described with the data. A *P* value < 0.05 was considered significant. All data analyses and statistical computations were conducted with SAS software, version 9.3 or higher (SAS Institute, Inc., Cary, NC) or GraphPad Prism, version 7.02 (GraphPad Software, LaJolla, CA).

## RESULTS

### Study population, experience with DVI, and tolerability.

A total of 105 Tanzanian volunteers (Figure 1) met the criteria (Supplemental Tables 2 and 3) and were enrolled. Ninety-three received either PfSPZ Vaccine (*n* = 63) or NS (*n* = 30) (Table 1). The remaining 12 volunteers participated as infectivity controls in the subsequent challenge portion (CHMI) of the protocol and are not part of the analyses in this article. There were no significant differences between participants in any age group for age, height, weight, or body mass index (BMI) (*P* > 0.05 for all comparisons, one-sided analysis of variance (ANOVA) (Table 2). All 93 volunteers received all scheduled immunizations. One volunteer in group 5b received only a partial vaccine dose with the second immunization; all other volunteers received the complete 0.5-mL injection at all time points.

Of 273 total injections, 234 were completed with a single injection (85.7%); 225 of these (96.2%) were assessed as simple to perform by the nurse performing the injection. In volunteers aged ≤ 5 years old, DVI was successful on the first attempt in 25 of 39 for first dose, 26 of 36 for second dose, and 31 of 36 for the third dose. The option to establish intravenous access with an intravascular catheter was used for 12 first injections in volunteers ≤ 5 years old, and one first injection in a 6–10-year old. An intravenous catheter was used only once for a second injection in an infant and was not used during the third round of injections, consistent with evidence of a learning curve with the DVI technique in infants and young children.

Pain from DVI was assessed in volunteers aged 6–45 years (groups 1a, 1b, 2a, 2b, 3a, and 3b); 157 of 161 injections (97.5%) were associated with mild or no pain (Supplemental Table 5).

**Safety.** A global summary of solicited AEs is provided in Table 3. Among the 63 volunteers who received 183 doses of

PfSPZ Vaccine, one Group 1b adult volunteer who received  $1.8 \times 10^6$  PfSPZ reported three solicited local AEs (tenderness and pain after first dose and tenderness after second dose); all were Grade 1 in severity and resolved within 2 days. No solicited local AEs were reported among the 30 volunteers after receiving 90 doses of NS. Solicited systemic AEs were detected after 3/183 injections of PfSPZ and 0/90 injections of NS. All 16 AEs occurred in a single Group 2b volunteer (age 11 years) who received a dose of  $1.8 \times 10^6$  PfSPZ. All AEs were Grade 1 except elevated temperatures, which were Grade 2. Symptoms included chills and feverishness after two of the three injections with fatigue, headache, malaise, and elevated temperature of 38.5°C after all three injections. The AEs occurred 12–24 hours after each immunization and resolved within 24 hours. This individual had no change in total white blood cell or absolute neutrophil counts or biochemistry values 2 days after each immunization but did have a mild decline in total lymphocyte counts that did not go below the lower limit of normal on Day 2 after each immunization. No other vaccine recipient experienced a systemic solicited AE. The local AEs in the one adult and the systemic AEs in the one adolescent are delineated in Figure 2. No significant differences were found between vaccinees and placebo recipients with respect to systemic or local event rates whether assessed as overall rates or specific rates for each type of AE (*P* = 0.60 for all by Barnard's test).

Twenty of 63 vaccinees (31.7%) experienced 30 unsolicited AEs (0.48/individual) during the 28 days after each immunization (Table 3). All unsolicited AEs were identified as unlikely related to administration of IP. Three unsolicited AEs were moderate (Grade 2) and one (the serious adverse event [SAE] described subsequently) severe (Grade 3) in severity; all others were mild (Grade 1). Ten of 30 controls (33.3%) experienced 13 unsolicited mild (Grade 1) AEs (0.43/individual) during this period. Two episodes of fever in one volunteer (Group 4a, age 3 years receiving  $4.5 \times 10^5$  PfSPZ), occurring 14 and 19 days after the first dose of NS, were determined to be possibly related to study product during the blinded safety assessment. Details of the unsolicited AEs can be found in Supplemental Table 6.

One SAE was reported in a 2-year-old volunteer (Group 4a) receiving  $4.5 \times 10^5$  PfSPZ Vaccine who was hospitalized with multiple injuries after she was struck by a motorcycle. She subsequently recovered and completed participation in the study.

Four volunteers developed parasitemia during the immunization period. Three infections in adults were detected retrospectively by qPCR (Supplemental Table 7); these individuals were TBS negative throughout this period of the study. Two of the three cases were Pm infections and one case was Pf, the latter confirmed by genotyping to differ from the NF54 strain of Pf used in the vaccine (Supplemental Table 8). The two individuals with Pm were determined retrospectively to be positive by qPCR at the time of first immunization and remained positive during the entire 16-week immunization period until treated after the third immunization. The individual with Pf infection was negative at the time of the first immunization, developed Pf infection before the second immunization, and remained positive until treated after the third immunization. All three volunteers were treated with artesunate–amodiaquine once the polymerase chain reaction results were known. A fourth volunteer, from Group 5 (ages 6–12 months), who received NS, was positive by qPCR for Pf genotypically distinct

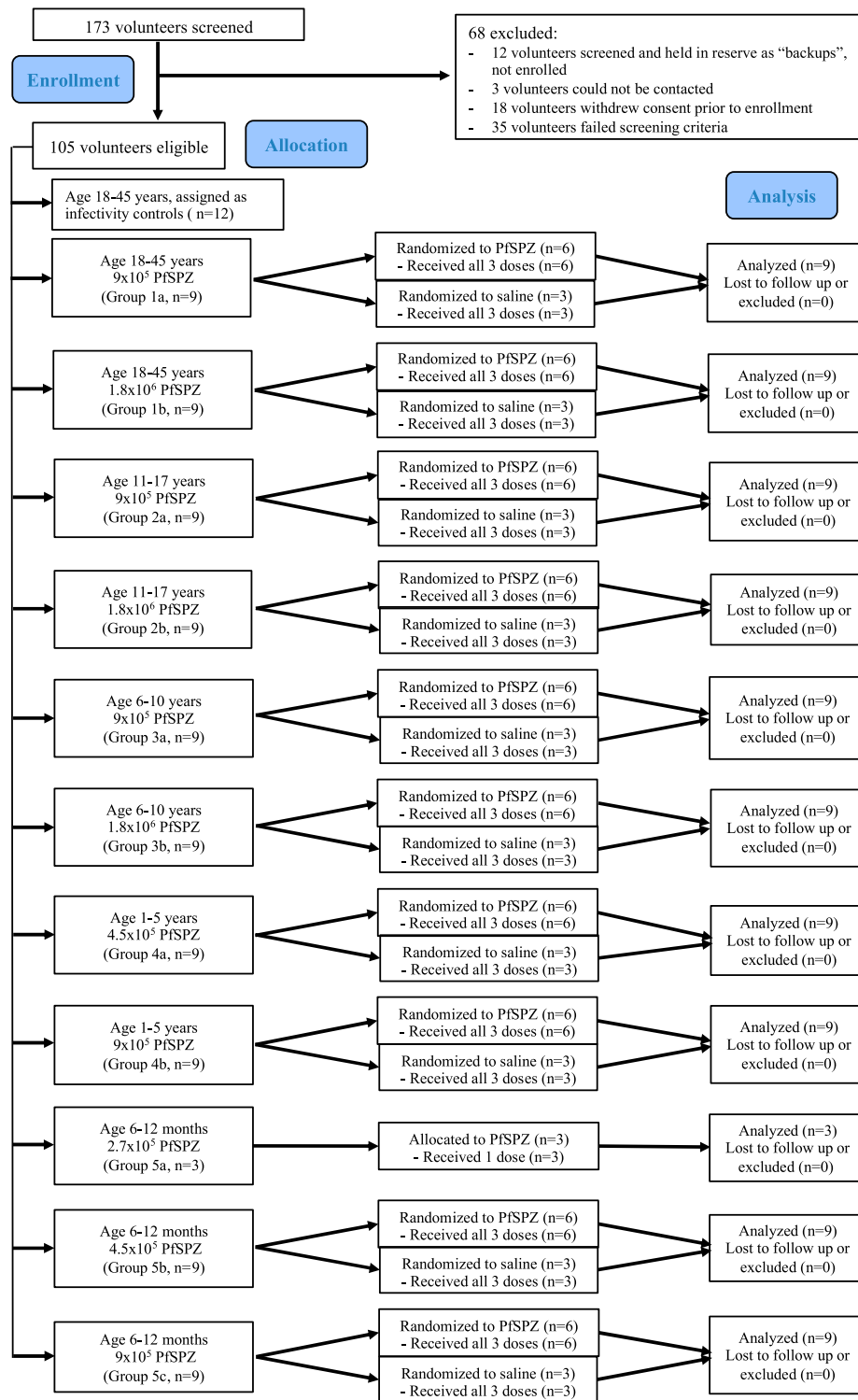


FIGURE 1. Volunteer participation (CONSORT 2010 Diagram). Once allocated, no volunteers were removed, lost to follow-up, or excluded from analysis. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

from the NF54 vaccine strain (Supplemental Tables 7 and 8) before the first and second immunizations; parasitemia in this infant was detected in real time by TBS before the second immunization, leading to immediate treatment with artemether-lumefantrine (Supplemental Table 7). No signs of illness were reported by the mother for this infant, who continued in the trial.

No clinically significant laboratory abnormalities were attributed to PfSPZ (Supplemental Table 9). The most commonly identified abnormalities listed in order of prevalence included anemia, lymphopenia, and leukopenia, with no difference in frequency across age groups or between vaccine recipients and NS controls in each age group. Two volunteers experienced a Grade 3 laboratory abnormality: an isolated

TABLE 2  
Volunteer characteristics

		Group 1 (18–45 years)				Group 2 (11–17 years)				Group 3 (6–10 years)			
		9 × 10 <sup>5</sup> (N = 6)		1.8 × 10 <sup>6</sup> (N = 6)		9 × 10 <sup>5</sup> (N = 6)		1.8 × 10 <sup>6</sup> (N = 6)		9 × 10 <sup>5</sup> (N = 6)		1.8 × 10 <sup>6</sup> (N = 6)	
		Years	Years	Years	Years	Years	Years	Years	Years	Years	Years	Years	Years
Age	Units	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median
	Mean (SD)	23.5 (5.7)	24.2 (5.3)	28.7 (7.9)	23.9 (4.8)	11.8 (1.0)	13.2 (1.3)	12.2 (1.3)	7.8 (1.5)	7.8 (1.5)	8	7.8 (1.5)	8
	Median	22	24	30	23	12	13	12	8	8	8	8	8
	(min, max)	(20, 35)	(18, 33)	(19, 38)	(18, 36)	(11, 13)	(11, 15)	(11, 14)	(6, 10)	(6, 10)	(6, 10)	(6, 10)	(6, 10)
Sex	Male	4 (66.7%)	4 (66.7%)	5 (83.3%)	7 (58.3%)	3 (50.0%)	2 (33.3%)	3 (50.0%)	3 (50.0%)	3 (50.0%)	4 (66.7%)	3 (50.0%)	3 (50.0%)
	Female	2 (33.3%)	2 (33.3%)	1 (16.7%)	5 (41.7%)	3 (50.0%)	4 (66.7%)	3 (50.0%)	3 (50.0%)	3 (50.0%)	2 (33.3%)	3 (50.0%)	3 (50.0%)
Race	African	6 (100%)	6 (100%)	6 (100%)	12 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)
Height (cm)	Mean (SD)	163.2 (5.0)	166.5 (10.4)	166.3 (8.0)	157.1 (9.0)	139.9 (15.3)	153.5 (9.8)	145.4 (9.2)	123.2 (9.2)	123.2 (9.2)	122.0 (7.8)	123.3 (7.1)	123.3 (7.1)
	Median	164.0	166.0	166.0	158.3	139.8	154.3	149.8	125.3	125.3	120.8	123.0	123.0
	(min, max)	(154, 168)	(149, 178)	(153, 175)	(136, 167)	(118, 165)	(138, 165)	(134, 154)	(112, 137)	(112, 137)	(114, 135)	(114, 135)	(114, 135)
Weight (kg)	Mean (SD)	62.3 (8.1)	65.8 (11.2)	64.3 (3.3)	58.0 (8.4)	33.2 (10.9)	40.6 (9.4)	37.8 (9.3)	23.3 (5.4)	23.3 (5.4)	21.2 (4.5)	22.9 (2.4)	22.9 (2.4)
	Median	62.5	67.0	65.0	57.5	31.5	41.0	39.3	24.0	24.0	20.5	22.5	22.5
	(min, max)	(53, 70)	(51, 80)	(60, 68)	(44, 75)	(21, 53)	(28, 55)	(25, 51)	(17, 29)	(17, 29)	(16, 29)	(20, 27)	(20, 27)
BMI	Mean (SD)	23.6 (4.1)	23.7 (2.9)	23.4 (2.6)	23.6 (3.8)	16.6 (2.6)	17 (2.4)	17.6 (2.7)	15.2 (1.9)	15.2 (1.9)	14.1 (1.2)	15.1 (1.7)	15.1 (1.7)
	Median	23.5	23.6	23.1	23.4	16.1	16.5	17.3	15.1	15.1	14.1	15.2	15.2
	(min, max)	(19, 30)	(20, 28)	(20, 27)	(19, 31)	(14, 20)	(15, 20)	(14, 22)	(13, 18)	(13, 18)	(12, 16)	(14, 16)	(14, 16)

		Group 4 (1–5 years)				Group 5 (6–11 months)			
		4.5 × 10 <sup>5</sup> (N = 6)		9 × 10 <sup>5</sup> (N = 6)		2.7 × 10 <sup>5</sup> (N = 3)		4.5 × 10 <sup>5</sup> (N = 6)	
		Years	Years	Years	Years	Months	Months	Months	Months
Age	Units	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median	Mean (SD)	Median
	Mean (SD)	3.3 (1.0)	3.3 (1.0)	2.7 (1.9)	3.0 (1.3)	7.3 (1.5)	7.3 (0.8)	8.7 (1.5)	9.3 (1.9)
	Median	3	3	3	3	7	8	8	10
	(min, max)	(2, 5)	(2, 5)	(1, 5)	(2, 5)	(6, 9)	(6, 8)	(7, 11)	(6, 11)
Sex	Male	2 (33.3%)	2 (33.3%)	3 (50.0%)	3 (50.0%)	1 (33.3%)	4 (66.7%)	2 (33.3%)	1 (16.7%)
	Female	4 (66.7%)	4 (66.7%)	3 (50.0%)	3 (50.0%)	2 (66.7%)	2 (33.3%)	4 (66.7%)	5 (83.3%)
Race	African	6 (100%)	6 (100%)	6 (100%)	6 (100%)	3 (100%)	6 (100%)	6 (100%)	6 (100%)
Height (cm)	Mean (SD)	99.7 (7.4)	99.7 (7.4)	91.6 (16.0)	96.1 (7.1)	66.0 (2.6)	67.7 (2.5)	69.9 (3.8)	66.0 (4.2)
	Median	99.3	99.3	92.0	93.8	65.0	68.3	71.8	66.5
	(min, max)	(92, 111)	(92, 111)	(72, 113)	(89, 107)	(64, 69)	(63, 70)	(63, 73)	(59, 71)
Weight (kg)	Mean (SD)	14.3 (1.6)	14.3 (1.6)	12.3 (3.9)	13.3 (1.4)	7.0 (1.0)	8.0 (0.9)	8.4 (1.2)	7.8 (1.2)
	Median	14.5	14.5	11.3	13.0	7.0	8.0	8.8	7.5
	(min, max)	(12, 16)	(12, 16)	(9, 18)	(12, 15)	(6, 8)	(7, 9)	(7, 10)	(7, 10)
BMI	Mean (SD)	14.5 (1.4)	14.5 (1.4)	14.5 (1.3)	14.5 (1.4)	16.0 (1.2)	17.5 (2.4)	17.2 (1.6)	18 (2.8)
	Median	14.6	14.6	14.4	14.5	16.6	17.9	17.2	16.9
	(min, max)	(13, 16)	(13, 16)	(13, 17)	(13, 16)	(15, 17)	(14, 20)	(15, 20)	(16, 23)

CHMI = controlled human malaria infection.

TABLE 3  
Global adverse event (AE) summary

	Vaccine (N = 63)		Placebo (N = 30)	
	All AEs	Possibly, probably, or definitely related AEs	All AEs	Possibly, probably, or definitely related AEs
No. of volunteers with at least one solicited AE within 7 days of immunization (%)	2 (3.2%)	2 (3.2%)	0 (0.0%)	0 (0.0%)
Total no. of solicited AEs (maximum severity grade)	19 (Grade 2)*	18 (Grade 2)*	0 (NA)	0 (NA)
No. of volunteers with a solicited Grade 3 AE (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
No. of volunteers with at least one solicited local AE	1 (1.6%)	1 (1.6%)	0 (0.0%)	0 (0.0%)
Total no. of local AEs (maximum severity grade)	3 (Grade 1)	3 (Grade 1)	0 (NA)	0 (NA)
No. of volunteers with at least one solicited systemic AE (%)	1 (1.6%)	1 (1.6%)	0 (0.0%)	0 (0.0%)
Total no. of systemic AEs (maximum severity grade)	16† (Grade 2)*	15† (Grade 2)*	0 (NA)	0 (NA)
No. of volunteers with at least one unsolicited AE within 28 days of immunization (%)	23 (36.5%)	0 (0.0%)	10 (33.3%)	1 (3.3%)
Total no. of unsolicited AEs within 28 days of immunization (maximum severity grade)	34 (Grade 3)	0 (NA)	11 (Grade 1)	2 (Grade 1)
No. of volunteers with an unsolicited Grade 3 AE (%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
No. of volunteers experiencing an SAE (%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total no. of SAEs (maximum severity grade)	1 (Grade 3)	0 (NA)	0 (NA)	0 (NA)

SAE = serious adverse event.

\* The only Grade 2 AE was elevated temperature.

† All solicited systemic AEs occurred in a single individual.

occurrence of lymphopenia in a Group 1b adult volunteer 28 days after the first dose of  $1.8 \times 10^6$  PfSPZ in association with a concomitant viral infection, and neutropenia in a Group 5b volunteer receiving  $4.5 \times 10^5$  PfSPZ determined to have benign ethnic neutropenia. Both abnormalities resolved without sequelae.

**Antibody responses to Pf.** Antibodies against PfCSP by ELISA, PfSPZ by aIFA, and PfSPZ by aISI in sera taken before immunization and 2 weeks after last vaccine dose are shown in Supplemental Table 10, Figure 3, and Supplemental Figure 1 for vaccinees and Supplemental Table 11 for controls.

In the PfCSP ELISA, volunteers were considered to have made a positive response if their net optical density (OD) 1.0 (Figure 3A–C) and OD 1.0 ratio (Supplemental Figure 1A–C), calculated, respectively, by subtracting or dividing by the prevaccination antibody OD 1.0, were  $\geq 50$  and  $\geq 3.0$ , respectively. By these criteria, 59/60 vaccinees developed antibodies to PfCSP. The only volunteer who did not have a

positive response to immunization was a 20-year old who received the  $9.0 \times 10^5$  PfSPZ regimen (Supplemental Table 10). The overall highest responses were in 6–10-year olds who received  $1.8 \times 10^6$  PfSPZ and had a median net OD 1.0 of 34,793 and median net OD 1.0 ratio of 15,515 (Supplemental Table 8). Only 2/30 of the NS controls, both infants, developed antibodies to PfCSP (Supplemental Table 11).

In the aIFA, volunteers with a net arbitrary fluorescence unit (AFU)  $2 \times 10^5$  of  $\geq 150$  (Figure 3D–F) and a ratio of post- to pre-AFU  $2 \times 10^5$  of  $\geq 3.0$  (Supplemental Figure 1, panels D–F) were considered to have made a positive response. By these criteria, 57/60 volunteers made a positive response to immunization. The three volunteers who did not make a positive response were 1 year, 7 months, and 9 months of age at the time of first injection and received the  $9.0 \times 10^5$ ,  $4.5 \times 10^5$ , and  $9.0 \times 10^5$  PfSPZ dosing regimens, respectively (Supplemental Table 10). As with the PfCSP ELISA, the overall highest

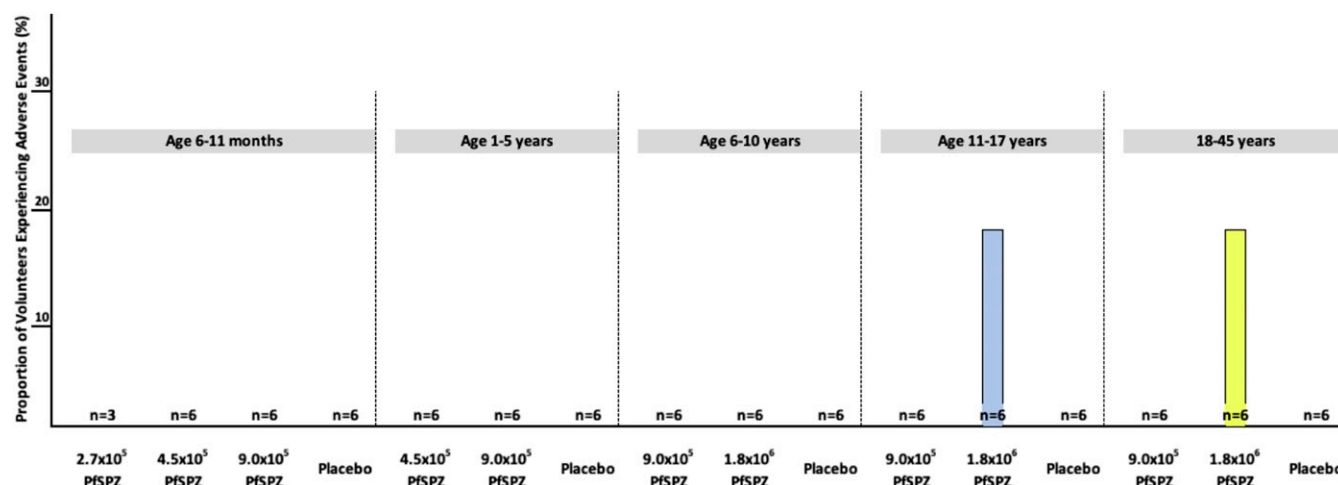


FIGURE 2. Proportion of volunteers experiencing solicited adverse events (AEs) during the 7 days after each immunization. Ninety-one of 93 volunteers injected at least once experienced no solicited AEs during the 7 days after each immunization. One volunteer experienced three Grade 1 local AEs (yellow bar) and one volunteer experienced Grade 2 temperature elevation ( $38.5^\circ\text{C}$ ) after each immunization accompanied by mild (Grade 1) chills, fatigue, headache, and malaise (blue bar). These AEs are further described in the text. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

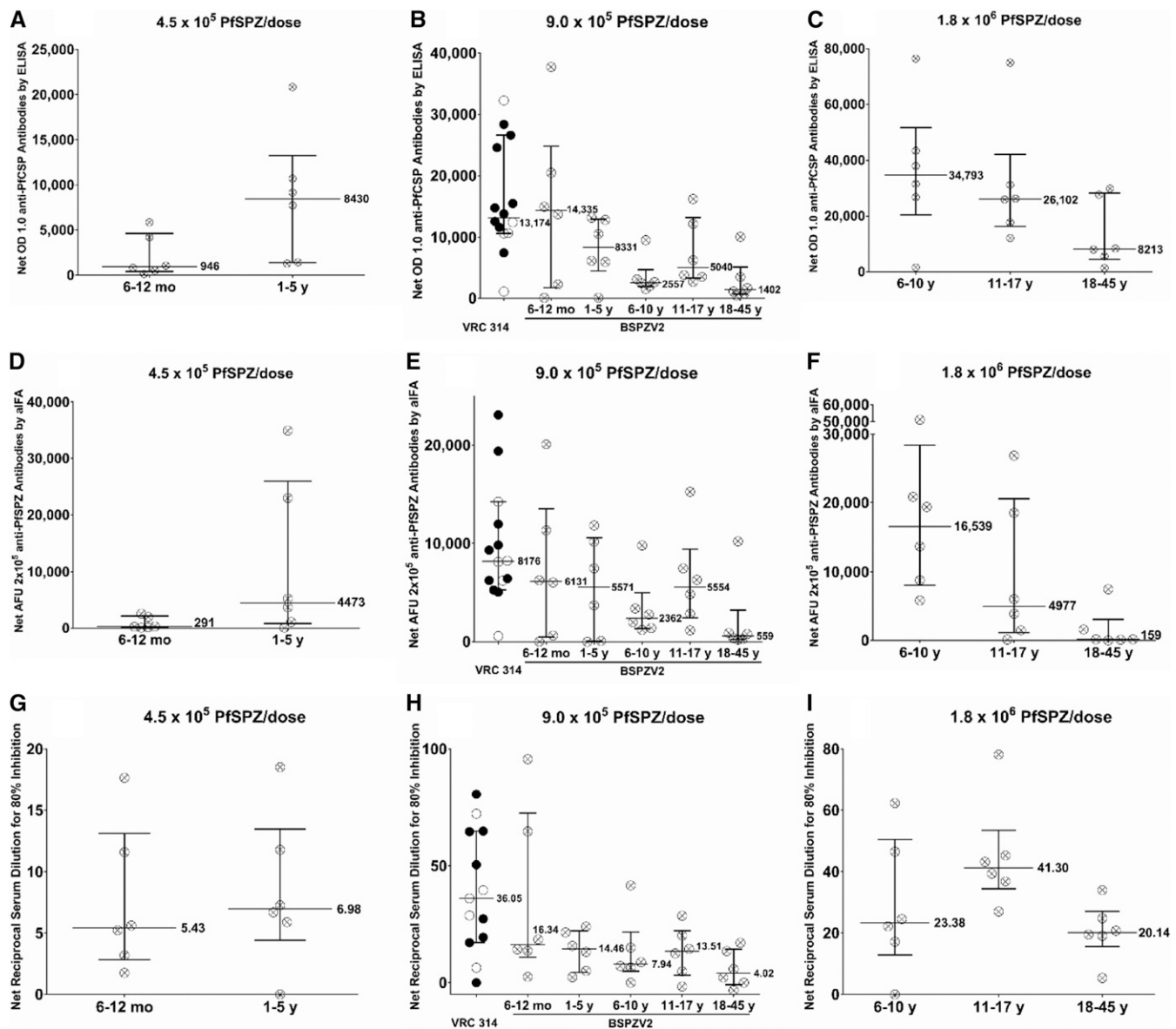


FIGURE 3. Difference between postimmunization and preimmunization antibody results. Antibody assay results by dose in *Plasmodium falciparum* circumsporozoite protein enzyme-linked immunosorbent assay (A–C), immunofluorescence assay (D–F), and inhibition of sporozoite invasion (G–I) assays. Results were obtained by subtracting preimmunization values from the values obtained from sera drawn 2 weeks after the third dose. For the  $9.0 \times 10^5$  *Plasmodium falciparum* sporozoite dose (B, E, and H), previously assessed results from clinical trial VRC 314, conducted in the United States with the same dosage regimen and in the same laboratory with the same assays as in the BSPZV2 trial assays, are shown as a comparison. For the VRC 314 trial, the filled in circles indicate protected volunteers and the empty circles the unprotected volunteers. Medians with interquartile ranges are shown.

responses were in 6–10-year olds who received the  $1.8 \times 10^6$  PfSPZ regimen and had a median net AFU  $2 \times 10^5$  of 20,099 and median net AFU  $2 \times 10^5$  ratio of 16,539 (Supplemental Table 10). None of the NS controls developed antibodies to PfSPZ by aIFA (Supplemental Table 11).

In the automated inhibition of sporozoite invasion assay (ISI), volunteers with a net ISI activity of  $\geq 10\%$  (Figure 3G–I) and ratio of post to pre-ISI activity of  $\geq 3.0$  (Supplemental Figure 1, panels G–I) were considered positive. By these criteria, 37/60 volunteers had a positive response to immunization. The only group with a 100% response rate (6/6) was the 11–15-year olds who received the  $1.8 \times 10^6$  PfSPZ regimen. This group also had the highest median net 80% ISI activity (41.3) and median net 80% ISI activity ratio (26.8). Only 2/30 of

the NS controls developed antibodies to PfSPZ by aISI, a 38 and a 13-year old (Supplemental Table 11).

Adults (18–35 years), teenagers (11–15 years), and older children (6–10 years) received the  $1.8 \times 10^6$  PfSPZ dosage regimen, and these age groups and the younger children (1–5 years) and infants (7–11 months) received the  $9.0 \times 10^5$  PfSPZ regimen; the younger children and infants also received the  $4.5 \times 10^5$  the PfSPZ regimen. We, therefore, assessed the effect of age on immunogenicity. This is shown graphically using net values in Figure 3 and for ratios in Supplemental Figure 1 and in Supplemental Tables 10 and 11. For the  $1.8 \times 10^6$  PfSPZ dosage regimen, the adults had the lowest antibody responses by all assays. The older children had the highest responses in the PfCSP ELISA and aIFA and teenagers

for the aISI. For the  $9.0 \times 10^5$  PfSPZ dosage regimen, the adults had the lowest median net OD 1.0, median net AFU  $2 \times 10^5$ , and median net 80% ISI responses, and the lowest ratios for PfCSP ELISA and aISI; infants had the highest responses for all of these assays. For the median AFU  $2 \times 10^5$  ratio, the teenagers had the best response. For the  $9.0 \times 10^5$  PfSPZ dosage regimen, the median OD 1.0 and median OD 1.0 ratios in the PfSPZ ELISA were 10.2 and 165.7 times higher in the infants as compared with the adults. For the aIFA, they were 11.0 and 3.3 times higher and for aISI, they were 4.1 and 6.3 times higher, respectively (Figure 3, Supplemental Figure 1, Supplemental Table 10). Because of the small sample sizes ( $N = 6$ ) and variability within each group, the differences did not quite reach the level of statistical significance.

Adults, teenagers, and older children received the  $1.8 \times 10^6$  or  $9.0 \times 10^5$  regimens. For all three assays, the response to the  $1.8 \times 10^6$  regimen was higher than the response to the  $9.0 \times 10^5$  regimen (Figure 3, Supplemental Figure 1, Supplemental Table 10). Younger children and infants received the  $9.0 \times 10^5$  and  $4.5 \times 10^5$  regimens. For all three assays, the response to the  $9.0 \times 10^5$  PfSPZ regimen was higher than the response to the  $4.5 \times 10^5$  regimen in infants. However, for the younger children, this was not the case (Figure 3, Supplemental Figure 1, Supplemental Table 10).

In previous clinical trials with PfSPZ Vaccine in adults in the United States with no previous exposure to malaria, there has been a significant correlation between the three different antibody assays<sup>14–17</sup> in sera taken 2 weeks after the last dose of vaccine. In this clinical trial, we assessed the correlation between the assays for sera taken 2 weeks after the third (last) dose of vaccine from 60 volunteers. There was a significant correlation between the results of the PfCSP ELISA and the aISI ( $R^2 = 0.45$ ,  $P < 0.0001$ ). The correlations between PfCSP ELISA and aIFA ( $R^2 = 0.05$ ,  $P = 0.085$ ) and aIFA versus aISI ( $R^2 = 0.01$ ,  $P = 0.40$ ) were not significant.

Having demonstrated that median antibody responses in Tanzanian infants were consistently higher than in Tanzanian adults, we compared the Tanzanian responses to those in adults in the United States who received three doses of  $9.0 \times 10^5$  PfSPZ (Figure 3B, E, and H). Median antibody responses in U.S. adults and Tanzanian infants were, respectively: 1) 13,174 and 14,335 for PfCSP ELISA, 2) 8,176 and 6,131 for aIFA, and 3) 36.05 and 16.34 for aISI.

**T-cell responses to PfSPZ.** T-cell responses to PfSPZ were assessed by polychromatic flow cytometry on cryopreserved PBMCs acquired before immunization, 2 weeks after the first dose of PfSPZ Vaccine and 2 weeks after the third dose of vaccine in subjects who received three doses of  $9.0 \times 10^5$  PfSPZ of PfSPZ Vaccine (Figure 4 and Supplemental Table 12). Following the first dose of PfSPZ Vaccine, 18–45-year olds, 11–17-year olds, and 6–10-year olds all had significant increases in the frequency of cytokine-producing memory CD4 T cells compared with the prevaccination time point. However, only the 6–10-year-old group had a 100% response rate to the vaccine. In addition, the responses in the 6–10-year-old group were significantly higher than those in the 1–5-year-old and 6–11-month-old groups, but not significantly different than the responses in the other groups (as assessed by the Kruskal–Wallis test with Dunn's correction for multiple comparisons). Following the third vaccination, only the 1–5-year olds had a significant increase over the prevaccination

time point. Infants did not have a significant increase in CD4 T-cell responses at any time point. At no time point were PfSPZ specific memory CD8 T-cell responses detected over background in any group.

## DISCUSSION

Before this study, aseptic, purified, cryopreserved PfSPZ-based products (Sanaria PfSPZ Vaccine, PfSPZ Challenge, and PfSPZ-CVac) had only been injected into adults.<sup>13–19,27,29,35–41</sup> This was the first study to assess the safety, tolerability, feasibility, and immunogenicity of any PfSPZ-based product in adolescents, children, or infants.

Because no licensed vaccine against an infectious agent is administered by DVI, there was initially a concern in the vaccinology community about the safety, tolerability, and feasibility of administering PfSPZ-based products by DVI to adults. Clinical trials in the United States, Germany, Spain, Mali, Tanzania, and Gabon established that rapid administration by DVI of PfSPZ products in 0.5 mL of diluent through a 25-gauge needle was safe, extremely well tolerated, straightforward, and protective.<sup>16,18,27,38,39,41</sup> After establishing the safety, tolerability, and feasibility of PfSPZ administration by DVI in adults, there was still concern that DVI administration of PfSPZ might be problematic in younger age groups as no preventative vaccine has ever been administered by intravenous injection (IV)/DVI to these age groups. This concern was not borne out by the findings of the present study.

There were no significant differences in solicited AEs between vaccinees in any age group ( $N = 63$ ) and corresponding controls ( $N = 30$ ) who received NS ( $P = 0.6$ ). Furthermore, there were no differences in AEs between different dosage regimens, no differences between age groups, and no differences between the first, second, and third immunizations. PfSPZ Vaccine was extremely well tolerated. We have no explanation for the symptoms and elevated temperature experienced by the 11-year-old girl after all three injections, which resolved within 24 hours of each immunization. This has not occurred in any subject in any other clinical trial of PfSPZ Vaccine; we will monitor for this pattern in future trials.

Moreover, the administration process itself (DVI) was extremely well tolerated. Six- to 45-year olds were questioned about pain after each injection. Direct venous inoculation was associated with mild or no pain for 157 of 161 injections (97.5%); 129 of 161 injections (80.1%) were associated with no pain (Supplemental Table 5).

There was no significant difference in the feasibility of administration of PfSPZ (or NS placebo) to adults, adolescents, 6–10-year olds, or 1–5-year olds. Administration was achieved with the first needle stick in 53/54 (98%) administrations in 18–45-year olds, 51/54 (94%) administrations in 11–17-year olds, 47/54 (87%) administrations in 6–10-year olds, and 47/54 (87%) administrations in 1–5-year olds (Supplemental Table 5). Administration success with one needle stick decreased to 35/57 (61%) injections in infants (Supplemental Table 5). However, in infants, the learning curve of the nurses administering the vaccine was rapid. In infants, DVI was successful on the first attempt in nine of 21 (43%) for first dose, 11 of 18 (61%) for second dose, and 15 of 18 (83%) for the third dose. The option to establish intravenous access with an intravascular catheter was used for 12 first injections (nine infants), but only once for a second injection (one infant)



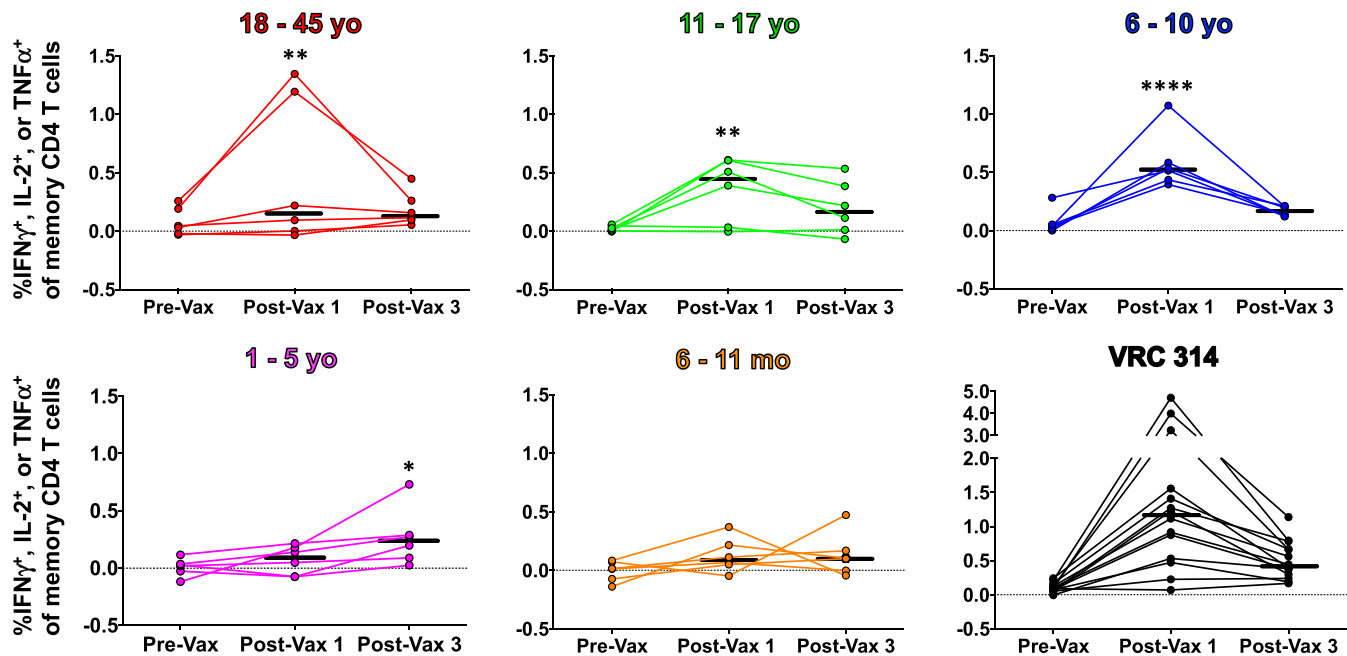


FIGURE 4. *Plasmodium falciparum* sporozoites (PfSPZ)-specific memory CD4 T-cell responses pre- and postvaccination. Percent of memory CD4 T cells in the blood expressing interferon gamma (IFN-γ) interleukin 2 (IL-2) or tumor necrosis factor alpha (TNF-α) at preimmunization or 2 weeks after the first and third doses of PfSPZ Vaccine ( $9.0 \times 10^5$ ). Results are the percentage of cytokine-producing cells after incubation with PfSPZ minus the percentage of cytokine-producing cells after incubation with vaccine diluent (medium with 1% human serum albumin). Bars indicate median values within each group. Differences within each age group between pre- and postvaccination groups were assessed by two-way ANOVA with Dunnett's correction for multiple comparisons. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ . Previously measured results from clinical trial VRC 314<sup>17</sup> conducted in the United States with the same dosage regimen and the same assay conducted in the same laboratory as for the BSPZV2 trial assays are shown as a comparison. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

and was not used during the third round of immunizations, consistent with evidence of a learning curve with the technique when administering to infants.

The levels of antibodies to PfCSP by ELISA were 31 times lower in adults in Mali than in U.S. adults and 4.3 times lower in adults in Tanzania,<sup>20</sup> who received the exact same immunization regimen.<sup>18</sup> We hypothesized that this was due to immunoregulation after long-term exposure to Pf infections and that in malaria-endemic areas, antibody responses would be higher in children and infants who had less exposure to Pf than in adults with long-term exposure. Naturally acquired immunity may also have affected the viability of the sporozoites, and this also is most highly developed in adults with long-term exposure. However, the levels of antibody preimmunization and in the placebo controls were quite low, arguing against this explanation for reduced immunogenicity (Supplemental Tables 10 and 11). Results from this study are consistent with the hypothesis that reduced immune responses in semi-immune African as compared with non-immune American adults was due to immunoregulation after long-term exposure to Pf infections, and that in malaria-endemic areas antibody responses would be higher in children and infants who had less exposure to Pf than in adults with long-term exposure. The median antibody responses by PfCSP ELISA, PSPZ aIFA, and PfSPZ aISI were highest in infants and lowest in adults (Figure 3), and the antibody responses in infants were comparable with the antibody responses seen in adults in the United States who received the identical immunization regimen (Figure 3). However, differences among age groups did not quite reach the level of statistical significance because of the small sample size and

the variance. Ongoing studies will establish whether this age effect is consistent and significant.

T-cell responses against PfSPZ have been demonstrated in malaria-naïve adults immunized with PfSPZ Vaccine in the United States<sup>14,16</sup> and in a previous study of PfSPZ Vaccine in adults in Tanzania,<sup>20</sup> but they were much lower in Tanzania than in the United States after administration of the same immunization regimen. In this trial, there were no CD8 T-cell responses against PfSPZ detected. However, significant increases in peripheral CD4 T-cell responses were seen in all age groups except infants after in vitro stimulation with PfSPZ (Supplemental Table 12). The median adult responses were about six times lower than they were after immunization with the same regimen in the United States (Figure 4).<sup>17</sup> Consistent with all of our trials, the best responses in adults, 11–17-year olds, and 6–10-year olds were seen after the first dose of PfSPZ Vaccine<sup>14–17</sup> with the highest responses in 6–10-year olds. We have previously hypothesized that the reason peak T-cell responses are highest after the first dose is that after the first dose the functionally important T cells are resident in the liver and, thus, are not detected in the periphery.<sup>14</sup> However, after the third dose of PfSPZ Vaccine, the only significant results were in 1–5-year olds; this was the best response recorded for this age group. Perhaps, because of immunological immaturity, it took longer to prime 1–5-year olds, and if they were administered more priming doses earlier, they would have better responses. Infants did not have any evidence of induction of T-cell responses. Such poor T-cell responsiveness in infants has also been observed following hepatitis B and oral polio vaccinations.<sup>42,43</sup> This is likely based on the fact that T-cell repertoires in neonates and infants are



skewed toward Th2-type responses.<sup>44–48</sup> Since PfSPZ Vaccine is thought to rely primarily on T-cell responses to mediate protection,<sup>14,15,17,49</sup> the T-cell studies may indicate that children, but not infants, will be protected by the immunization regimen (three doses at 8-week intervals) used in this study. We are now exploring priming regimens in which multiple doses of PfSPZ are administered during the first week,<sup>27</sup> and this may prove to be a more powerful method of priming and could thereby overcome the poor T-cell responsiveness we have identified in infants. However, we recognize that we will be breaking new ground here, as to our knowledge, there are no data in infants for any vaccine that this can be done. In addition, we will explore the impact of booster doses during the first or second years of life, and we are developing an adjuvant that may be useful.

By establishing the safety, tolerability, and feasibility of administration of PfSPZ Vaccine to children and infants, this trial was an important prelude to clinical trials in more than 300 infants being conducted in Kenya (ClinicalTrials.gov NCT02687373) and Equatorial Guinea (ClinicalTrials.gov NCT02859350). It also supported our long-term plans to use PfSPZ Vaccine in mass vaccination programs (MVPs) to focally eliminate malaria. Because high population coverage will be needed to halt transmission, any vaccine intended for this purpose must be extremely safe, easy to administer, and minimally painful for the vaccinee, in all age groups. As there was no difference in the rate of AEs or laboratory abnormalities between any vaccine group and placebo recipients and because 97.2% of volunteers old enough to evaluate pain experienced no pain or only mild pain during administration, PfSPZ Vaccine appeared in the present study to be highly suitable for use in MVPs.

The results demonstrated that 6–10-year olds and 1–5-year olds have the highest CD4 T-cell responses after the first and third doses of PfSPZ Vaccine, respectively. These findings raise hope that the vaccine will be more protective in children than it was in adults in Mali.<sup>18</sup> This will soon be assessed in 1–12-year olds in Gabon. However, because no T-cell responses were identified in infants, we are concerned about infants' immunological capacity to mount protective T-cell responses after this immunization regimen of PfSPZ Vaccine. This is now being assessed in the Kenya study, and if it holds up, we may have to alter the immunization regimen (number of PfSPZ per dose, number of doses, and interval between doses). If this is not successful, we would likely initiate immunization only after the first year of life. PfSPZ Vaccine is intended to be used in MVPs to halt transmission of Pf and not in a routine infant immunization program (Expanded Program for Immunization). In such MVPs, we would cover the infants with antimalarial drugs until they reached 1–2 years of age when immunizations with PfSPZ Vaccine would begin.

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Disclosures: Sanaria, Inc. manufactured PfSPZ Vaccine and Protein Potential LLC is affiliated with Sanaria. Sanaria was the sponsor of the clinical trial. Thus, all authors associated with Sanaria or Protein Potential have potential conflicts of interest.

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# **Controlled Human Malaria Infections in Tanzania and Equatorial Guinea: The impact of malaria pre-exposure on diagnosis and multiplication rate of *P. falciparum* parasites**

This is working manuscript, which is currently not submitted for review at a peer review journal

## Abstract

Controlled human malaria infections (CHMI) provide the opportunity to study immunological responses to infection and parasite-host interaction in a controlled setting. We used the data from 159 individuals who participated in CHMI conducted in two different malaria endemic settings to characterise the impact of malaria pre-exposure on the parasites' multiplication rate (PMR). Compared to malaria-naïve German volunteers, in Tanzanian and Equatorial Guinean volunteers, the parasite's ability to replicate in the blood was significantly reduced. Among these volunteers, the PMR was especially low if they recently cleared parasitaemia from an ongoing *P. falciparum* single infection or *P. falciparum* and *P. malariae* co-infection. We used CHMI data to investigate the impact of a repeated homologous CHMI on the PMR of *P. falciparum* parasites during a follow up second CHMI conducted in individual volunteers. A reduction in PMR was only observed if during the first CHMI blood-stage parasites were detected. Furthermore, the reduction in PMR resulted in an overall lower peak parasitaemia and subsequently less symptomatic volunteers. This data indicates the development of stage-specific parasite growth reducing immune effector mechanisms.

## Keywords

Controlled human malaria infection (CHMI), quantitative polymerase chain reaction (qPCR), thick blood smear (TBS) microscopy, *Plasmodium falciparum*, PfSPZ Challenge, diagnosis, immunity

## Introduction

Despite a significant global decline in malaria prevalence and reduction of mortality between 2010 and 2017, malaria remains a global public health issue with an estimated 219 million cases which resulted in 435 000 deaths in 2017 (1). Control interventions such as the distribution of long-lasting insecticidal nets and indoor residual spraying programs as well as widespread and rapid access to diagnostic testing and treatment have proven to be successful. But with the ultimate goal of malaria eradication new approaches, including safe and effective vaccines are needed (2). Controlled human malaria infection (CHMI), the deliberate inoculation of volunteers with *Plasmodium* spp. sporozoites by mosquito bite or syringe and needle is increasingly being used to assess the efficacy of experimental malaria vaccines and contributes to an acceleration in malaria vaccine development (3).

During CHMI, the volunteers are closely monitored for signs and symptoms of malaria. Parasitaemia in whole blood is rigorously monitored and volunteers are treated before they develop high parasitaemia levels and severe symptoms. Historically, the appearance of blood-stage parasitaemia was diagnosed using thick blood smear (TBS) microscopy while recently the more sensitive quantitative polymerase chain reaction (qPCR) is used as a primary diagnostic test in CHMI. Several CHMI conducting research sites have discussed the use and in particular the advantages of qPCR-based monitoring of parasitaemia in malaria-naïve (4–8) and pre-exposed individuals (7, 9, 10). In summary, the implementation of qPCR in CHMI improves safety while reducing the clinical burden and costs without compromising the evaluation of protective efficacy for pre-erythrocytic vaccine candidates (4).

CHMI studies are not only useful for the evaluation of vaccine candidates but also provide the opportunity to study parasite-host interactions in a highly controlled setting. Although, conducting CHMIs in malaria endemic regions would allow earlier assessment of vaccine

efficacy in target populations and provide insight into the immunological responses associated with naturally acquired immunity (NAI) (11), the vast majority of CHMI has been conducted in malaria-naïve volunteers (12). In the past, the challenge to conduct CHMI in Africa was attributed to lack of high security facilities rearing *P. falciparum*-infected *Anopheles* mosquitoes (13). This obstacle had been overcome by Sanaria's aseptic, purified, cryopreserved *P. falciparum* sporozoites (PfSPZ Challenge) that are highly infectious (14). In 2012, a study in Tanzania showed for the first time that inoculation of malaria pre-exposed volunteers with PfSPZ Challenge is safe, well tolerated and highly infective (13). These findings were later confirmed in studies conducted in Kenya (10) and Gabon (15, 16) and Mali (unpublished) where adult volunteers from malaria endemic regions were successfully infected with PfSPZ Challenge.

Although the exact mechanisms of NAI are still poorly understood, it is thought that repeated infection with *P. falciparum* leads to the development of NAI mediated by a broad range of antibodies directed against blood-stage antigens which have the ability to limit parasite replication (17–19). The parasite multiplication rate (PMR), the fold change in number of parasites in the blood over one life cycle, has been used to characterize NAI in recent studies (10, 15, 20, 21). PMR can be calculated based on the qPCR-derived parasite densities measured during the pre-patent periods of CHMI (22).

In this study we describe parasitological data obtained from CHMI studies conducted in Tanzania and Equatorial Guinea to characterize the impact of malaria pre-exposure on the parasites' multiplication rate and ability to diagnose *P. falciparum*.

## Material and Methods

### Controlled Human Malaria Infection

Parasitological and clinical data presented in this publication was obtained from CHMI used to assess PfSPZ Vaccine efficacy in five studies conducted in Tanzania and Equatorial Guinea. Standardized CHMI protocols were used in all five studies with 3200 aseptic, purified, cryopreserved PfNF54 *P. falciparum* sporozoites (PfSPZ Challenge) administered by direct venous inoculation. The time point of sporozoite inoculation (herein referred to as CHMI) was defined as day 0. The period of active parasitemia monitoring, the days post CHMI when volunteers were admitted to the ward (herein referred to as ward period), varied between the different studies. For BSPZV1 (Clinical Trials.gov: NCT02132299), samples were collected twice daily from day 9 to 14 and once daily from day 15 to 21 after PfSPZ Challenge inoculation. For BSPZV2 (Clinical Trials.gov: NCT02613520) and BSPZV3 (Clinical Trials.gov: NCT03420053), samples were collected twice daily from day 8 to 14 and once daily from day 15 to 20 after PfSPZ Challenge inoculation. For EGSPZV2 (Clinical Trials.gov: NCT02418962), samples were collected twice daily from day 8 to 14 and once daily from day 15 to 20 after PfSPZ Challenge inoculation followed by sample collection on alternate days until day 28. During the EGSPZV3 study (Clinical Trials.gov: NCT03590340) additional samples were collected at day 6 and 7, before the sampling schedule of the EGSPZV2 study was followed. At day 28 (BSPZV1, BSPZV2, BSPZV3 and EGSPZV3) or day 56 (EGSPZV2) all TBS microscopy negative volunteers were treated with antimalarial drugs. Adverse events during the CHMI ward period were assessed for relationship to *P. falciparum* infection and considered related if the event was within 3 days before and 7 days after TBS microscopy was first positive.

### **Collection, processing and storage of blood samples for malaria diagnosis**

For all parasitological analysis, including TBS microscopy, qPCR and backup storage, 2 mL of EDTA-anticoagulated whole blood was collected and aliquoted. The aliquot for TBS microscopy was immediately processed and the aliquot for qPCR analysis was frozen at  $-80^{\circ}\text{C}$  until further processing. DNA was extracted manually from 180  $\mu\text{L}$  whole blood and eluted with 50  $\mu\text{L}$  Elution Buffer using Quick-DNA Miniprep Kit (Zymo Research, Irvine, CA).

### **Thick blood smear microscopy**

In all five studies, TBS microscopy was performed following the same standard operating procedure as previously described (13). Briefly, 10  $\mu\text{L}$  of blood was placed uniformly on a 1 cm x 2 cm area of the slide, air dried, and stained with Giemsa, pH 7.2. Routine assessment of asymptomatic volunteers required the microscopist to read 6 passes (1 cm each) with the 100x objective, which equals to approximately a blood volume of 0.5  $\mu\text{L}$ . If the volunteer was symptomatic, a total of 24 passes of the slide was read corresponding to 2  $\mu\text{L}$  of blood. A blood smear was declared positive when one reader saw two parasites in 0.5  $\mu\text{L}$  of blood and the presence of parasites was independently confirmed by a second reader. This corresponds to a theoretical limit of detection (LOD) of 4 parasites/ $\mu\text{L}$ . Microscopists were trained and evaluated before each study and only highly qualified microscopists were allowed to read slides during CHMI studies.



## Molecular diagnostic using qPCR

Parallel to TBS microscopy, qPCR was used to determine and quantify parasitaemia. qPCR analysis was conducted on site and in real-time during the CHMI ward period. Since the trials were conducted in regions where non-*falciparum Plasmodium* species are endemic, our approach included both, a pan-species *Plasmodium* spp. and a *P. falciparum*-specific assay. For the first two trials (BSPZV1 and BSPZV2) we used two independent qPCR assays. A probe-based duplex assay, amplifying the pan-species *Plasmodium* spp 18S rDNA (Pspp18S) (23) and as an internal control the human *rnasep* gene (HsRNaseP) was applied to the samples, followed by screening with an EvaGreen-based assay detecting the *P. falciparum*-specific telomere-associated repetitive element 2 (PfTARE2) (24). For the following studies (BSPZV3, EGSPZV2 and EGSPZV3) the diagnostic qPCR procedure was simplified and workload reduced by switching the *P. falciparum*-specific target to the acidic terminal sequence of the var genes (PfvarATS) (24). This allowed us to use a single qPCR assay for screening of *Plasmodium* spp. and *P. falciparum* parasites and therefore increased sample-to-result turnaround by reducing the qPCR time significantly. All parasite densities are expressed as number of parasites per mL blood. *P. ovale* spp. and *P. malariae* parasites were identified as described elsewhere (25). Detailed information concerning molecular diagnostic assays used is provided in Table S1.

## Data management and analysis

The Cq values were obtained from the Bio-Rad CFX96 software and in case of the BSPZV1, BSPZV2 and EGSPZV2 studies, transferred to a Microsoft Access database designed for storage and analysis of qPCR data. For the BSPZV3 and EGSPZV3 studies, the Cq values were transferred and analysed to the ELIMU-MDx platform (*manuscript in preparation*). Parasite Multiplication Rate (PMR) was modelled using a linear model fitted to log10-transformed qPCR data as previously published (22). PMR was calculated for all volunteers that developed blood-stage parasitaemia which lasted for at least two 48-hour cycles. Parasite densities based on the *P. falciparum*-specific qPCR assays, PfTARE2 or PfvarATS, were used to calculate PMRs. Data was analysed and plots created using R version 3.5.1 based on packages *dplyr*, *ggpubr*, *gridextra* and *scales*.

## Results

### **Data collection from malaria pre-exposed volunteers who developed blood-stage parasitaemia upon CHMI**

In two different malaria-exposed populations, one in East Africa (Bagamoyo, Tanzania) and one in Central-West Africa (Malabo, Equatorial Guinea), the efficacy of the PfSPZ Vaccine was assessed using CHMI. In this publication we combined the volunteers who developed blood-stage parasitaemia from both clinical trial sites to describe the CHMI associated blood-stage growth dynamics of *P. falciparum* in malaria-exposed volunteers. In total, 90 Tanzanian volunteers participating in three clinical trials and 69 volunteers from Equatorial Guinea participating in two clinical trials were included in the analysis. 18 volunteers from Germany were used as a reference group for malaria-naïve volunteers (Table 1). Notably, all forty-six Tanzanian and five out of six Equato-Guinean volunteers who served as placebo controls in the PfSPZ Vaccine studies developed blood stage parasitaemia detectable by qPCR providing an overall infectivity rate of 98.0% (50/51). Sub-microscopic infections, parasitaemia not seen by thick blood smear microscopy, were observed in 9.4 % (15/159) of volunteers. Malaria treatment was initiated 15.6 days (median and range: 15 [10-28 days]) after CHMI resulting in a time period of 6.1 days (median and range: 5.5 [1.4-21.0 days]) of pre-patent period which was used to study the parasite growth dynamics.

## Diagnosis of *P. falciparum* during CHMI using TBS microscopy and qPCR

Primary study outcome was parasitaemia measured by TBS microscopy which was used to initiate treatment and therefore terminate the CHMI. The qPCR-derived parasitaemia at CHMI endpoint (TBS positivity) was compared between the German, Tanzanian and Equatorial Guinean volunteers (Fig 1A). Compared to Tanzania and Germany, significantly higher parasitaemia levels were detected in volunteers from Equatorial Guinea. The significantly higher parasitaemia levels at TBS positivity in Equatorial Guinea possibly reflects the lower level of experience of microscopists at this recently established CHMI site. Volunteers in Tanzania or Equatorial Guinea had median parasite densities of 46'893 parasite/mL (47 parasites/ $\mu$ L) at time of TBS positivity with more than 80% of volunteers having parasitaemia levels greater than 20'983 parasite/mL (21 parasites/ $\mu$ L) (Fig 1B). This lower limit of detection (LOD) of TBS microscopy is in the range of published LODs for experienced microscopists (26), but higher than the theoretical LOD of 2000-4000 parasite/mL (2-4 parasites/ $\mu$ L) reported for this TBS microscopy protocol (5, 27). A weak correlation between parasite densities determined by TBS microscopy and qPCR was observed (Fig 1C) which is most likely explained the low parasitaemia levels assessed. The Bland-Altman analysis revealed higher parasite quantities derived from qPCR compared to TBS microscopy (Fig 1D). Parasitaemia levels derived from the qPCR assay are as double as high compared to the parasitaemia levels obtained from TBS microscopy (TBS/qPCR ratio: 0.52 with 95% CI: -1.19 - 2.22). *Bejon et al* demonstrated that large numbers of parasites are lost during preparation of the microscopy slides and concluded that this limits the sensitivity, and leads to underestimation parasite density by microscopy (28). On the contrary, qPCR-based measurement could overestimate parasite densities, for example if schizonts, containing multiple merozoites each carrying a complete haplotype, are amplified. The contribution of schizont stages to the total estimates of parasite densities is described to be low for *P. falciparum* (29) as well as *P. vivax* (30).

## **The importance of molecular parasitaemia monitoring in pre-exposed volunteers participating in CHMIs**

The benefits of using qPCR for monitoring parasitaemia levels over time becomes especially apparent if applied to populations with previous exposure to malaria. Given the high proportion of sub-microscopic malaria infections, only highly sensitive qPCR assays have the potential to ensure that volunteers are negative for malaria parasites before enrolled into CHMI. In our clinical trial sites, a considerable proportion of volunteers were found to be positive for malaria parasites prior to CHMI (Table 2). All of them were successfully treated and tested negative before they received the challenge strain. Proportions of volunteers positive for malaria parasites in Equatorial Guinea and Tanzania were found at 13.2% (18/136) and 4.4% (5/113), respectively. *P. falciparum* was the most common malaria species, followed by *P. malariae* and *P. ovale* spp. Peak parasitaemia levels were low with a geometric mean of 618 parasites/mL (0,618 parasites/ $\mu$ l), resulting in 73.9% (17/23) sub-microscopic infections (Table 2). The same issue of detectability of low parasitaemia levels also applies to CHMI initiated infections caused by the PfSPZ Challenge strain PfNF54. Unlike malaria-naïve volunteers, people with pre-existing NAI have the ability to control parasite multiplication, which can lead to sub-microscopic infections and therefore only will be identified by more sensitive qPCR assays. The ability to control the parasite replication over a long period of time is demonstrated with six volunteers from Equatorial Guinea (Figure 2). All six volunteers developed blood-stage parasitaemia early after CHMI, on average after 9.25 days (range: 8-15 days), followed by a high variability in temporal blood stage parasitaemia. The volunteers were left untreated until day 56 post CHMI and never developed a positive TBS microscopy result and no clinical signs or symptoms indicative of an ongoing malaria infection. The parasite densities remained low throughout the two months of follow-up, with a median peak par-

asitaemia of 5005 parasites/mL (range: 129-37'169 parasites/mL). On average, only one third of the total 25 bleeding time points assessed were positive (range: 8-72%).

### **Parasite multiplication rates in Tanzanian and Equato-Guinean volunteers**

The period between the appearance of blood-stage parasites, detected by qPCR, and treatment initiation, after detection by TBS microscopy, was used to calculate the PMRs. In the case of volunteers from Tanzania and Equatorial-Guinea, this period lasted on average 6 days which translates into three 48-hour blood stage cycles of *P. falciparum* and therefore allows to estimate the PMR robustly.

Since the PfSPZ Vaccine, acting as pre-erythrocytic stage vaccine, had no significant impact on the PMR compared to placebo controls (Wilcoxon test  $p=0.8$ ), we decided to combine vaccinated volunteers and placebo controls. A significantly reduced PMR was observed among Tanzanian (median [range]: 5.5 [0.8-42.9]) and Equato-Guinean (median [range]: 5.5 [0.6-21.2]) volunteers compared to malaria-naïve German volunteers (median [range]: 10.5 [2.0-46.1]) (Fig 3A). Interestingly, no difference between the two populations from East and Central-West Africa was observed. A subset of Tanzanian volunteers were genotyped for red blood cell polymorphisms, including sickle cell trait, alpha thalassemia and Glucose-6-phosphate dehydrogenase (G6PD) deficiency (31). Using this genotyping data, we compared Tanzanian volunteers which were wildtype for all three RBC polymorphism (wildtype controls,  $n=33$ ) with volunteers carrying heterozygous alpha thalassemia trait only (heterozygous alpha thalassemia,  $n=24$ ). The volunteers with heterozygous alpha thalassemia trait were wildtype for sickle cell trait and G6PD deficiency. No significant difference in *P. falciparum* PMR in volunteers carrying heterozygous alpha thalassemia trait was observed (Fig 3B). Stable and controlled HIV infection under ART treatment did not impact PMR either (Fig 3C). Prior to CHMI, all subjects were screened by TBS microscopy and qPCR to identify all natu-

rally acquired *Plasmodium* spp. infections. Six volunteers were found to be positive for *P. falciparum*, two carried an additional *P. malariae* co-infection. All six volunteers were successfully treated and cleared parasitaemia before being enrolled into CHMI. Importantly, a highly reduced PMR was observed in these six volunteers who were cleared from a field *P. falciparum* single infection or *P. falciparum* and *P. malariae* co-infection upon PfSPZ Challenge (Fig 3D).

### **The impact of two consecutive homologous CHMIs on the parasite multiplication rate**

In the BSPZV2 study, 16 Tanzanian volunteers were challenged twice with a time interval of seven to nine months between the two CHMIs. We grouped these participants according to the outcomes of the first CHMI. The first group consisted of seven subjects who did not develop blood stage parasitaemia during the first CHMI (“CHMI-1 blood-stage negative”). The second group included nine subjects who developed blood stage parasitaemia during the first CHMI (“CHMI-1 blood-stage positive”). The third group consisted of eleven subjects who served as CHMI controls and were challenged only once (“controls”). The “controls” group had a median PMR of 4.3 (range: 1.0-13.3) and 10 out of 11 volunteers were detected by TBS microscopy (Fig 4A, “controls”). While being blood-stage negative during the first CHMI, all seven volunteers developed blood-stage parasitaemia after the second CHMI, which was detectable in 6 out of 7 cases by TBS microscopy. The median PMR of 2.7 (range: 1.2-13.3) was not significantly different from the “controls” ( $p=0.79$ ) (Fig 4A, “CHMI-1 blood-stage negative”). Among the “CHMI-1 blood-stage positive” volunteers, during the first CHMI all nine subjects did develop parasitaemia levels detectable by TBS microscopy, while during the second CHMI only four volunteers reached parasite densities high enough to be detected by TBS microscopy. The PMR was significantly lower in all nine subjects after the second CHMI compared to the first challenge (Fig 4A, “CHMI-1 blood-stage positive”). The reduced

PMR during second CHMI resulted in significantly lower peak parasitaemia levels over the 28-day follow-up as compared to the first CHMI (Fig 4B, “CHMI-1 blood-stage positive”). Although not statistically significant, during the second CHMI, only one “CHMI-1 blood-stage positive” volunteer compared to four volunteers during the first CHMI, did show CHMI-related symptoms (Fig 4C, McNemar's test  $p=0.25$ ). No difference in the proportion of volunteers with CHMI-related symptoms among the “controls” and “CHMI-1 blood-stage negative” volunteers was observed. No significant differences are observed for the liver-to-blood inoculum (Fig 4D) and qPCR prepatent periods for “CHMI-1 blood-stage positive” volunteers between the first and second CHMI (Fig 4E). The parasite growth dynamics for each individual volunteer belonging to the “CHMI-1 blood-stage positive” group is shown in Supplementary Figure S1, separated by CHMI-1 and CHMI-2.



## Discussion

The use of PfSPZ Challenge for CHMI was the foundation for the establishment of CHMI platforms in malaria endemic regions. These platforms have the ability to contribute to an acceleration in malaria vaccine development in the target population. The standardized nature of CHMI, which includes exact time of infection, dose and strain of *P. falciparum*, also provides a unique opportunity to gain insight into the interaction of host genetics and/or NAI on the course and development of malaria infection.

We publish here blood-stage parasitaemia data determined by TBS microscopy and qPCR from 159 individuals who participated in CHMI conducted in two different malaria endemic settings. We conducted all qPCR analysis before, during or shortly after the CHMI studies on-site in Bagamoyo and Malabo. This turned out to be the most efficient and cost-effective way for monitoring these parameters in experimental malaria vaccine studies. An additional benefit is that by using two independent diagnostic procedures, TBS microscopy and qPCR, false positive TBS microscopy results can be prevented which could affect clinical trial results (32). Furthermore, CHMI studies conducted in malaria endemic regions are an accelerator for establishing laboratory infrastructure and provide an excellent training platform for human resource development for molecular diagnostics.

We used this data set to describe the impact of malaria pre-exposure on diagnosis of *P. falciparum* parasites. A significant proportion of volunteers did not develop parasitaemia levels high enough to be detected by TBS microscopy during CHMI. We describe six cases where CHMI-induced parasitaemia was stable at sub-microscopic levels for two months. Notably, on average, qPCR detected parasites in only one third of the total 25 bleeding time points analysed in these volunteers. This temporal variation in blood parasitaemia demonstrates also

the limitation if malaria prevalence assessments are based on a single blood sample analyzed during cross-sectional studies (33). Another challenge is the occurrence of naturally acquired malaria infections in volunteers before enrolment into CHMI. Almost three quarter of these cases identified presented themselves as asymptomatic, sub-microscopic infections and required qPCR assays to be detected.

We used the pre-patent period before malaria treatment commenced to calculate the PMR. Compared to malaria-naïve German volunteers, in both African populations, the parasite's ability to replicate was significantly reduced. We showed that recently cleared parasitaemia from an ongoing *P. falciparum* single infection or *P. falciparum* and *P. malariae* co-infection leads to a significantly reduced PMR during the CHMI study conducted.

We attempted to identify a genetic determinant of person-to-person variability in PMR by analysing the impact of the widely distributed heterozygous alpha thalassemia trait. While the exact mechanism remains unclear, a study conducted in two cohorts of children living at the coastal area of Kenya concluded that alpha thalassaemia is strongly protective against severe and fatal malaria (34). Among volunteer with heterozygous alpha thalassemia only, no reduction of *P. falciparum* growth rates *in vivo* was observed contradicting several *in vitro* experimental reports (35, 36). CHMI studies in combination with genotyping could provide a valuable platform to test old and new hypothesis on association between genetic traits and malaria as shown by our colleagues in Gabon (15) and as currently conducted in Kenya (37). The data on interaction between HIV and *P. falciparum* in the BSPZV3 study demonstrate the ability of CHMI also to provide the framework to study malaria in context of ongoing co-infections.

Molineux and colleagues used data collected between 1940 and 1963, when malaria therapy was used as a treatment for neurosyphilis, to study the impact of repeated malaria therapy (38). In addition to a reduction in parasite density, they also observed development of parasite

tolerance during a second malaria infection, suggesting the acquisition of anti-parasitic and antitoxic immunities. We used modern CHMI data to investigate the impact of a repeated homologous CHMI on the multiplication rate of *P. falciparum* parasites. A reduction in PMR can only be observed if during the first CHMI blood-stage parasites were detected. Repeated CHMI did not impact the liver-to-blood inoculum or qPCR prepatent period in those volunteers. This data is in agreement with the notion of stage-specific parasite growth reducing immune effector mechanisms in malaria. Furthermore, the reduction in PMR leads to an overall lower peak parasitaemia and subsequently less symptomatic volunteers confirming the link between parasitemia levels and clinical presentation.

In summary we conclude that malaria pre-exposure has a significant impact on *P. falciparum* asexual blood-stage growth. We showed a reduced PMR in malaria-pre-exposed individuals compared to malaria-naïve volunteers, and after a cleared parasitaemia from an ongoing malaria infection and finally after repeated homologous CHMI in volunteers who developed blood-stage parasitaemia during the first CHMI. Our data indicates the development of stage-specific parasite growth reducing immune effector mechanisms.

A consequence of this reduced asexual blood-stage growth due to malaria pre-exposure is that conventional diagnostic methods, such as microscopy and RDTs, fail to detect a substantial number of infections during CHMI as well as naturally acquired malaria infections.

**Table 1.** Collection of volunteers who developed blood-stage parasitaemia during CHMI

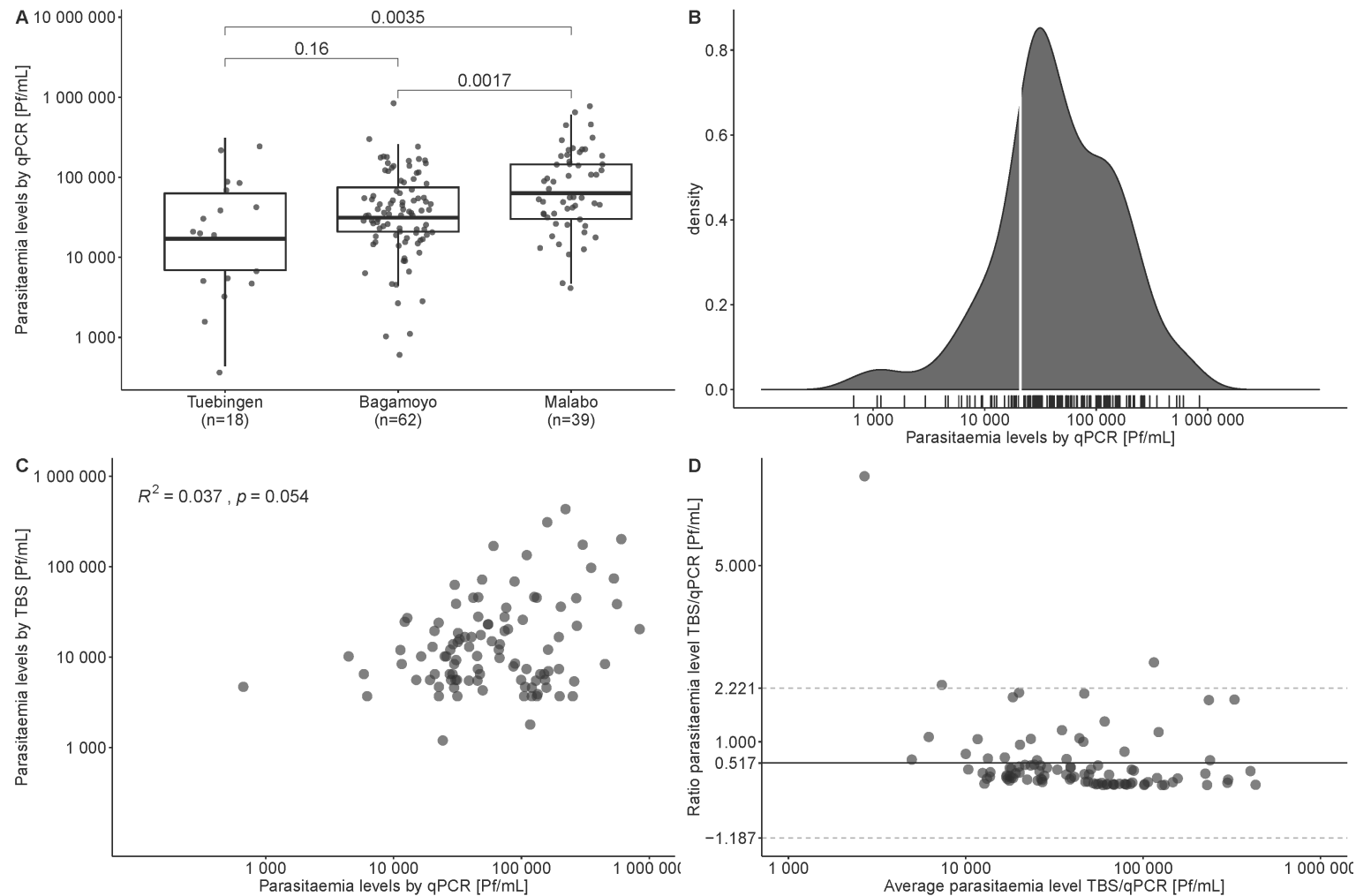
Country	Study	Sampling for parasitological analysis (days)	Number of volunteers with blood stage parasitaemia	TBS microscopy positive	Initiation of treatment (day) <sup>a</sup>	Period of blood stage parasitaemia (days) <sup>a</sup>
Tanzania	BSPZV1	9.0-28.0	57	52/57 (91.2%)	14 (10-19)	4.9 (1.4-20.0)
Tanzania	BSPZV2	8.0-28.0	21	19/21 (90.5%)	14 (12-20)	6.3 (4.1-20.0)
Tanzania	BSPZV3	8.5-28.0	12	9/12 (75.0%)	16 (12-18)	4.8 (2.5-19.0)
Equatorial Guinea	EGSPZV2	8.0-28.0	21	16/21 (76.2%)	16 (13-28)	6.5 (4.0-20.0)
Equatorial Guinea	EGSPZV3	6.0-28.0	48	39/48 (81.3%)	18 (14-28)	5.5 (4.5-21.0)
Total pre-exposed	-	-	159	144/159 (90.6%)	15 (10-28)	5.5 (1.4-21.0)
Germany	TUECHMI2	6.0-28.0	18	18/18 (100.0%)	11 (11-18)	5.0 (4.0-11.0)

<sup>a</sup>Median values and ranges are indicated.

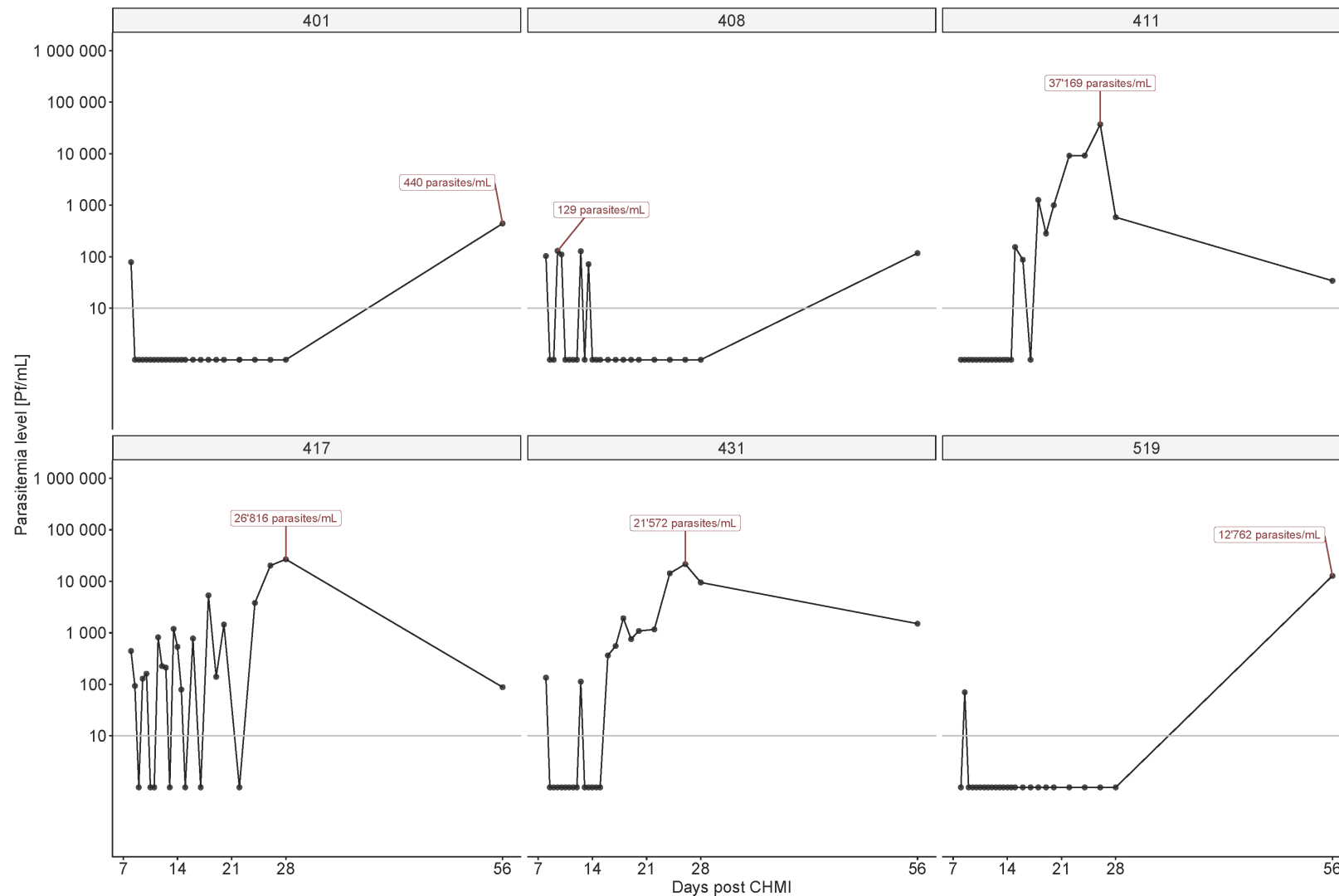
**Table 2.** Volunteers with naturally acquired malaria infections prior to CHMI

Subject ID (Study)	Country	Species	Peak parasitemia levels	TBS microscopy
001G1a (BSPZV2)	Tanzania	<i>P. falciparum</i>	35'290 parasites/mL	no
009G1a (BSPZV2)	Tanzania	<i>P. malariae</i>	200 parasites/mL	no
010G1a (BSPZV2)	Tanzania	<i>P. malariae</i>	4220 parasites/mL	no
032G2b (BSPZV3)	Tanzania	<i>P. falciparum</i>	140 parasites/mL	no
034G2b (BSPZV3)	Tanzania	<i>P. falciparum</i>	1'040'710 parasites/mL	yes
408G1a (EGSPZV2)	Equatorial Guinea	<i>P. falciparum</i> / <i>P. malariae</i>	3245 parasites/mL	no
416G1a (EGSPZV2)	Equatorial Guinea	<i>P. falciparum</i> / <i>P. ovale</i>	2407 parasites/mL	no
431G1a (EGSPZV2)	Equatorial Guinea	<i>P. falciparum</i> / <i>P. malariae</i>	1809 parasites/mL	no
519G1b (EGSPZV2)	Equatorial Guinea	<i>P. falciparum</i>	105 parasites/mL	no
525G1b (EGSPZV2)	Equatorial Guinea	<i>P. falciparum</i>	22'131 parasites/mL	no
530G1b (EGSPZV2)	Equatorial Guinea	<i>P. falciparum</i>	96 parasites/mL	no
031G1 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	16'310 parasites/mL	no
032G1 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	1310 parasites/mL	no
016G3 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	1840 parasites/mL	yes
134G3 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	227'420 parasites/mL	yes
148G4 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	27'100 parasites/mL	no
149G4 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	6'740'410 parasites/mL	yes
151G4 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	29'750 parasites/mL	no
154G4 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	14'440 parasites/mL	no
157G4 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	140 parasites/mL	yes
161G4 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	480 parasites/mL	no
162G4 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	536'560 parasites/mL	yes
173G4 (EGSPZV3)	Equatorial Guinea	<i>P. falciparum</i> *	700 parasites/mL	no

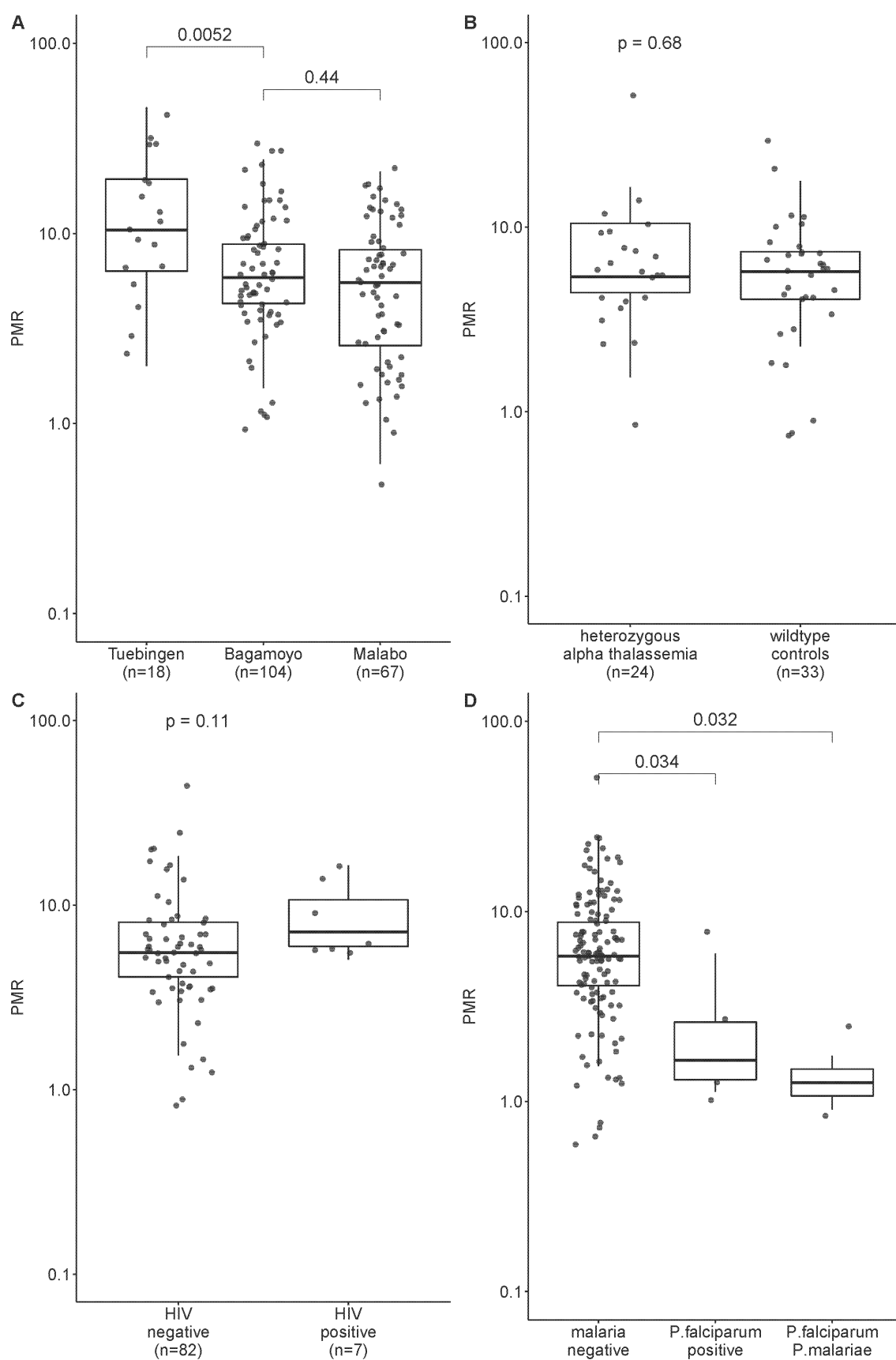
\* Confirmed *P. falciparum* infection possible co-infections with non-*P. falciparum* species were not identified.



**Figure 1. *P. falciparum* quantification using TBS microscopy and qPCR.** (A) Parasitaemia levels at TBs positivity compared between volunteers from three different clinical trial sites. Groups were compared using Wilcoxon-Mann-Whitney test. (B) Density plot of qPCR-derived parasite quantities at time of TBS microscopy positivity (median parasite densities of 46'893 parasite/mL). The white vertical line represents the parasitaemia level of 20'983 parasite/mL at which more than 80% of the volunteers were detected by TBS microscopy. (C) Correlation plot of parasite densities quantified by TBS microscopy and qPCR assay. Spearman's rank correlation coefficient is indicated. (D) Bland-Altman measurement of agreement between *P. falciparum* quantification using TBS microscopy and qPCR assay. The average ratio (solid black line) and the 95% confidence interval indicated by the dashed lines are shown.

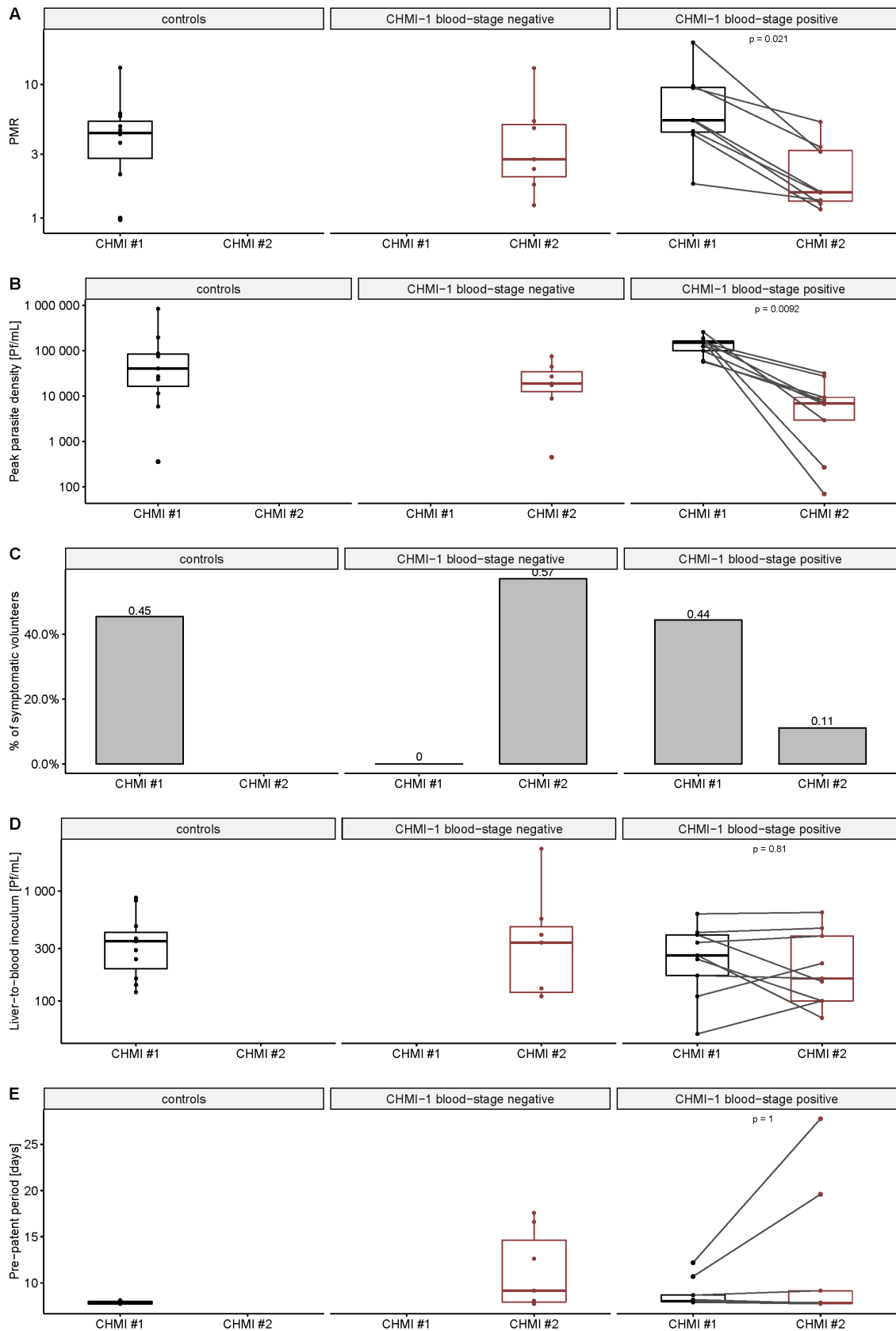


**Figure 2. Long lasting asymptomatic, sub-microscopic infections induced by CHMI.** Six Equato-Guinean volunteers developed asymptomatic, sub-microscopic infections after CHMI. Peak parasitaemia for each volunteer is indicated. The grey line represents the lower limit of qPCR positivity. Parasitaemia levels are plotted with an offset of 1 parasite/mL. A total of 25 individual blood samples were collected before malaria treatment was initiated at day 56 post CHMI.



**Figure 3. Parasite multiplication rates in Tanzanian and Equato-Guinean volunteers participating in CHMIs.** (A) PMRs compared between malaria-naïve volunteers (Germany) and volunteers from two malaria endemic regions (Tanzania and Equatorial Guinea). (B) Impact of heterozygous alpha thalassemia trait on PMR. (C) HIV status and PMR in Tanzanian volunteers. (D) Impact of naturally acquired malaria infection on PMR. All groups were compared using Wilcoxon-Mann-Whitney test.





**Figure 4. Impact of repetitive CHMI on parasite growth dynamics.** (A) PMR for all three groups calculated for first and second CHMI. (B) Peak parasitaemia levels for all three groups. (C) Proportion of symptomatic volunteers during first and second CHMI. (D) Liver to blood inoculum (LBI) between first and second CHMI. (E) qPCR pre-patent period (PPP) compared between first and second CHMI. Wilcoxon matched-pairs signed rank test was used to compare PMR, peak parasitaemia, LBI and PPP between CHMI 1 and CHMI 2.

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## **Competing interests**

LW Preston Church, Thomas L. Richie, Peter F. Billingsley and Stephen L Hoffman are salaried and full-time employees of Sanaria Inc, the developer and sponsor of Sanaria® PfSPZ Vaccine. They were not responsible for the collection, recording or entry of the parasitological data used in this study. The other authors declare that they have no competing interests.

## **Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article or submitted as supplementary data.

## **Ethics approval and consent to participate**

All trials were performed in accordance with Good Clinical Practices (GCP). The BSPZV1 (Clinical Trials.gov: NCT02132299) protocol was approved by institutional review boards (IRBs) of the IHI (Ref. No. IHI/IRB/No:02-2014), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/1691), the Ethikkommission Nordwest-und Zentralschweiz, Basel, Switzerland (reference number 261/13), and by the Tanzania Food and Drug Authority (Ref. No. TFDA 13/CTR/ 0003). The BSPZV2 (Clinical Trials.gov: NCT02613520) protocol was approved by IRBs of the IHI (Ref. No. IHI/IRB/ No: 32-2015), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/2049), and the Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland (reference number 15/104). The protocol was also approved by the Tanzania Food and Drug Authority (Auth. No. TZ15CT013). The BSPZV3 (Clinical Trials.gov: NCT03420053) protocol was approved

by IRBs of the IHI (Ref. No. IHI/IRB/ No: 32-2015), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/2049), and the Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland (reference number 15/104). The protocol was also approved by the Tanzania Food and Drug Authority (Auth. No. TZ15CT013). For the EG-SPZV2 (Clinical Trials.gov: NCT02418962) study ethical approval was obtained from the National Ethics Committee of Equatorial Guinea, the Ifakara Health Institute Institutional Review Board, Dar es Salaam, Tanzania, the MaGil Institutional Review Board in Maryland, USA, and the Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland. For the EGSPZV3 (Clinical Trials.gov: NCT03590340) study ethical approval was obtained from the National Ethics Committee of Equatorial Guinea, the Ifakara Health Institute Institutional Review Board, Dar es Salaam, Tanzania, the MaGil Institutional Review Board in Maryland, USA, and the Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland.

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## Supporting Information

**Table S1.** qPCR assays used for molecular malaria diagnostics

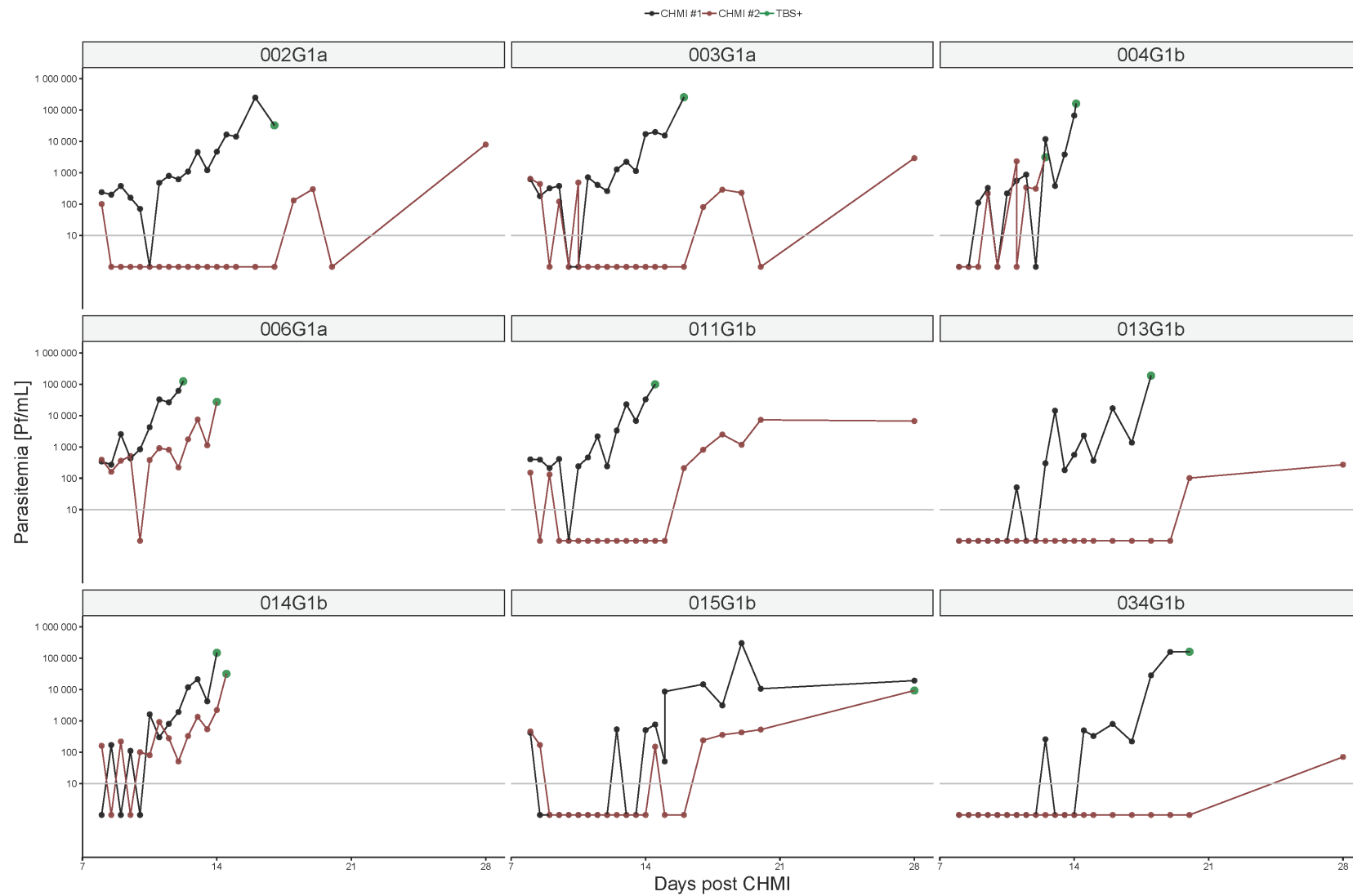
	PlasQ v 1.0	PlasQ v 2.0
Studies assay was applied	BSPZV1, BSPZV2	BSPZV3, EGSPZV2, EGSPZV3
Sample analysed	2655	5048
qPCR reagents	5x HOT FIREPol® Probe Universal qPCR Mix and 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biodyne, Tartu, Estonia)	Luna® Universal Probe qPCR Master Mix (New England Biolabs, Ipswich MA, USA)
qPCR analysis duration	Probe-based qPCR: 1h 51min EvaGreen-based qPCR: 2h 43min	Probe-based qPCR: 1h 29min
<i>P. falciparum</i> target	PfTARE2	PfvarATS
LOD of <i>P. falciparum</i> target	50 parasites/mL	20-50 parasites/mL
CV @ LOD for <i>P. falciparum</i> target	4.3%	3.2%
<i>Plasmodium</i> spp. target	18S rDNA	18S rDNA
LOD of <i>Plasmodium</i> spp. target	100-500 parasites/mL	100-200 parasites/mL
CV @ LOD for <i>Plasmodium</i> spp. target	3.0%	3.0%
Cq of internal control HsRNaseP (Mean/SD)	23.9 ± 1.4	25.7 ± 1.3
CV of HsRNaseP <sup>a</sup>	6.2%	5.0%

LOD: Limit of detection, defined as the lowest parasitaemia level of the PfIS which was amplified in all replicates.

CV: Coefficient of variance, calculated from the lowest detectable parasitaemia level of the PfIS standard across all individual standards for all five studies.

SD: Standard deviation

<sup>a</sup> Coefficient of variance was calculated based on Cq values of HsRNaseP for all samples.



**Figure S1.** Parasitemia growth dynamics of “blood stage positive” volunteers for first (black) and second (red) CHMI. Green dots indicate detection by TBS microscopy.

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# Chapter III

Development of high-throughput, field deployable tools  
for malaria surveillance

Chapter 3 includes three manuscripts describing the development of novel techniques which could support molecular malaria surveillance efforts in endemic regions.

First, in response to the emerging threat of *P. falciparum* strains lacking *pfhrp2* and/or *pfhrp3* genes and therefore evading detection by PfHRP2-based RDTs, we developed qPCR-based assay which is well suited for high-throughput identification of *pfhrp2* and *pfhrp3* gene deletions in *P. falciparum* isolates. The ability to detect “masked” *pfhrp2/3* deletions is probably the most interesting feature of our approach, because it will allow to study the epidemiology of *pfhrp2/3* deletions in malaria endemic regions with a high proportion of multiple strain co-infections.

The second manuscript describes our attempt to improve extraction of nucleic acids from used RDTs. We used an approach which enabled us to extract total nucleic acids, including DNA and RNA. This increased the overall sensitivity for the detection of *Plasmodium* spp. parasites in the small volume of blood which is available on an RDT stored at room temperature. Beside the detection and quantification of all malaria species, applying our protocol on thousands of RDTs, we can monitor *pfhrp2* and *pfhrp3* gene deletions in *P. falciparum*, anti-malarial drug resistance markers or even other blood borne pathogens circulating in malaria endemic areas.

The third manuscript describes our ELIMU-MDx platform. A web-based and open-source platform for storage, management and analysis of diagnostic qPCR data. In response to the vast amount of qPCR data generated during a series of CHMI studies with more than 250 individuals enrolled and conducted in Tanzania and Equatorial Guinea, we decided to design and build this platform which facilitates the rapid and automated analysis and interpretation of diagnostic qPCR data.

This chapter contains the following manuscripts:

Schindler *et al.* **A multiplex qPCR approach for detection of *pfhrp2* and *pfhrp3* gene deletions in multiple strain infections of *Plasmodium falciparum*.** *currently under review by Scientific Reports* 2019

Guirou and Schindler *et al.* **High Throughput Extraction and Analysis of Nucleic Acids from Rapid Diagnostic Tests for Molecular Surveillance of Malaria.** *manuscript to be submitted to Journal of Clinical Microbiology* 2019

Wehner and Schindler *et al.* **ELIMU-MDx: A Web-Based, Open-Source Platform for Storage, Management and Analysis of Diagnostic qPCR Data.** *submitted to BioTechniques* 2019

# **A multiplex qPCR approach for detection of *pfhrp2* and *pfhrp3* gene deletions in multiple strain infections of *Plasmodium falciparum***

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This manuscript is currently under review in *Scientific Reports*

## Abstract

The rapid and accurate diagnosis of *Plasmodium falciparum* malaria infection is an essential factor in malaria control. Currently, malaria diagnosis in the field depends heavily on using rapid diagnostic tests (RDTs) detecting circulating parasite-derived histidine-rich protein 2 antigen (PfHRP2) in capillary blood. *P. falciparum* strains lacking PfHRP2, due to *pfhrp2* gene deletions, are an emerging threat to malaria control programs. The novel assay described here, named qHRP2/3-del, is well suited for high-throughput screening of *P. falciparum* isolates to identify these gene deletions. The qHRP2/3-del assay can correctly identify *pfhrp2* and *pfhrp3* gene deletions in multiple strain co-infections, particularly prevalent in Sub-Saharan countries. Deployment of this qHRP2/3-del assay will provide cost efficient and rapid insight into the prevalence and potential spread of *P. falciparum* isolates that escape surveillance by RDTs.



## Introduction

Malaria is an infectious disease with an estimated 219 million cases globally and was responsible for 435'000 deaths in 2017. More than 90% of these malaria cases and deaths occurred in sub-Saharan Africa with *Plasmodium falciparum* as the most pathogenic malaria parasite species, accounting for 99.7% of malaria cases <sup>1</sup>.

Advances have been made in malaria control which have contributed to the decline in malaria prevalence observed worldwide with improved diagnostic tests and better access to malaria treatment contributing significantly to this development <sup>1</sup>. The rapid and accurate diagnosis and treatment of malaria cases is an essential factor in the control of malaria. Rapid diagnostic tests (RDTs) are becoming the most widely used method to diagnose malaria infections in the field with 245 million RDTs distributed worldwide in 2017 <sup>1</sup>. In sub-Saharan Africa, RDTs have almost completely replaced light microscopy for malaria diagnosis, with an estimated 75% of malaria tests conducted using RDTs in 2017 <sup>1</sup>. Malaria RDTs are based on an immuno-chromatographic assay using a lateral-flow device which allows the detection of malaria antigens in usually 5 to 15 µL of capillary blood <sup>2</sup>. RDTs provide results within 20 minutes and can be employed by inexperienced health workers operating in resource-limited settings <sup>3</sup>. RDTs recognizing circulating histidine-rich protein 2 (PfHRP2) for sensitive and specific detection of *P. falciparum* make up more than 90% of RDTs currently in use <sup>4</sup>. The relatively high abundance and stability of PfHRP2 in the blood of infected patients and expression by all *P. falciparum* erythrocytic stages make this antigen a valuable biomarker for malaria infection <sup>5</sup>. PfHRP3, a protein also expressed by *P. falciparum* with high level of structural similarity to PfHRP2, might be also recognized by some of the monoclonal antibodies used in the RDTs <sup>6</sup>.

RDTs are critical diagnostic tools for identifying symptomatic malaria infections; however, due to the reduced performance in infections with low parasite density, its use for the diagnosis of malaria infection in asymptomatic individuals is rather limited <sup>7</sup>.

Recent studies report on reduced diagnostic performance of PfHRP2-based RDTs which were attributed to genetic diversity of the *pfhrp2/3* genes <sup>6</sup>, differences in expression level of PfHRP2/3 antigen in parasite field strains <sup>8</sup> or isolates lacking *pfhrp2* and/or *pfhrp3* genes <sup>9</sup>. *P. falciparum* isolates lacking *pfhrp2* and/or *pfhrp3* genes are found around the world, with different proportions of the circulating *P. falciparum* population affected. The regions with the highest proportions of *P. falciparum* strains carrying *pfhrp2* deletions are South America and sub-Saharan Africa <sup>10</sup>. Since malaria control programmes depend on reliable diagnosis of malaria cases using RDTs, parasites lacking *pfhrp2/3* genes pose a threat to malaria control and local elimination efforts <sup>11</sup>.

The presence or absence of *pfhrp2/3* genes is usually determined by amplifying these genes by polymerase chain reaction (PCR). Several different (nested) PCR protocols have been published and a deletion is reported if there is no amplification of the *pfhrp2/3* genes in the presence of an amplification signal of a *P. falciparum* single copy gene, which serves as a reference gene <sup>12</sup>. There are methodological issues related to this approach which assumes identical PCR performance of the *pfhrp2/3* and the reference genes. Particularly at lower parasitaemia levels with a small number of DNA target molecules present, unavoidable stochastic effects can play a major role and might lead to false reporting of *pfhrp2/3* deletions. Additionally, conventional nested PCR methods are time consuming, requiring separate reactions for each target gene amplification as well as gel electrophoresis for visualization of the PCR products. Furthermore, none of the published methods detecting *pfhrp2/3* deletions can identify “masked” deletions in multiple strain infections with only

one out of several *P. falciparum* strains carrying a *pfhrp2* and/or *pfhrp3* gene deletion <sup>13</sup>. These limitations of recommended molecular monitoring methods could result in an underestimation of the prevalence of *P. falciparum* strains with *pfhrp2/3* deletions, especially in regions with high proportions of multiple strain co-infections.

This paper presents a novel, quantitative PCR-based method for detecting *pfhrp2* and *pfhrp3* gene deletions suitable for high throughput screening of *P. falciparum* isolates. The qHRP2/3-del (quantitative detection of *pfhrp2* and *pfhrp3* deletion) assay was developed as a multiplex assay, with the ability to amplify individually and specifically the *pfhrp2* and *pfhrp3* genes together with a single copy gene, the *P. falciparum* ribonucleotide reductase R2\_e2 (*pfprn2e2*) <sup>14</sup>, as an internal reference. The quantitative nature of the qHRP2/3-del assay provides the basis for estimating the proportions of *P. falciparum* strains carrying *pfhrp2* and *pfhrp3* deletions in regions with multi-clonal malaria infections.

# Results

## Design and evaluation of the novel qHRP2/3-del assay

We aimed at improving the detection of *pfhrp2* and *pfhrp3* gene deletions by developing a quantitative PCR-based assay able to detect and quantify *pfhrp2* and *pfhrp3* genes in a single reaction. Given the high nucleotide sequence similarity and the repetitive structure of the *pfhrp2* and *pfhrp3* genes, nucleotide regions serving as targets for primers and probes were limited (Supplementary file 1). The primer and probe combinations selected for our assay (Table 1) bind to a region spanning exon 1 and exon 2 of both genes to detect entire and partial gene deletions described to occur on known chromosome breaking points<sup>12</sup>. We designed a multiplex qPCR assay using three differently labelled TaqMan assays detecting the *pfhrp2* (PF3D7\_0831800) and *pfhrp3* (PF3D7\_1372200) genes with the single copy gene *pfmr2e2* (PF3D7\_1015800) as the internal control. The sequence alignment of the *pfhrp2* and *pfhrp3* genes highlighting the oligo binding regions is shown in Supplementary file 1.

The multiplexed assays correctly identify *P. falciparum* strains carrying known deletions of *pfhrp2* (PfDD2 strain) and *pfhrp3* (PfHB3 strain) as well as a strain without deletion (PfNF54 strain) (Fig 1A). The multiplexed assays show comparable characteristics in terms of sensitivity and qPCR performance. All three targets are detected in samples with parasitaemia as low as 1 parasite/ $\mu$ L and an inverse linear correlation between Cq values and parasite densities ranging from 1 to 10'000 parasites/ $\mu$ L was observed. The qPCR efficiencies were calculated as 85.7%, 98.8% and 98.4% for the amplification of *pfhrp2*, *pfhrp3* and *pfmr2e2*, respectively (Fig 1B). The qHRP2/3-del assay was next tested using purified DNA from eight culture adapted *P. falciparum* strains from Africa (Pf3D7, PfNF54, PfNF166.C8), South and Central America (Pf7G8, PfDD2, PfHB3), South East Asia (PfNF135.C10) and Papua New Guinea (PfFC27) with known deletion status of the *pfhrp2*

and *pfhrp3* genes (Fig 1C). The Cq values for amplification of *pfrnr2e2* were comparable between the eight strains amplified demonstrating its robustness and accuracy as internal control. No significant differences of Cq values for the *pfhrp2* gene and *pfhrp3* gene across the strains carrying the genes was observed. Sequence alignments of PfNF135.C10, Pf3D7, Pf7G8, PfNF54 and PfNF166.C8 did not reveal sequence variation in the oligo binding regions of *pfhrp2* (Supplementary file 2) or *pfhrp3* (Supplementary file 3) supporting these findings. DNA derived from five non-*falciparum* *Plasmodium* species (*P. ovale curtisi*, *P. ovale wallikeri*, *P. malariae*, *P. knowlesi*, *P. vivax*) was tested with the qHRP2/3-del assay and did not result in amplification of any target demonstrating the specificity for *P. falciparum* (Fig 1D). In summary, we developed a *P. falciparum*-specific multiplex qPCR assay that allowed the simultaneous amplification of the *pfhrp2*, *pfhrp3* and *pfrnr2e2* genes in a single reaction with high efficiency and ability to correctly identify *pfhrp2* and *pfhrp3* gene deletions.

### **Analysis of *P. falciparum* field strains with qHRP2/3-del assay**

The qHRP2/3-del assay was next tested using a collection of 254 *P. falciparum* isolates originating from East Africa, Central-West Africa and Latin America (Table 2). The infection status and parasitaemia levels were well established in these samples by using diagnostic qPCR assays deployed in the laboratories where the samples were obtained. The overall median parasitemia in these samples was 75.7 parasites/ $\mu$ L (IQR: 2.2-571.6), which is below the LOD of 100 parasites/ $\mu$ L for PfHRP2-based RDTs. First, the ability of the *pfrnr2e2* singly copy gene to serve as internal assay control and to quantify parasitemia levels was assessed. Out of the 254 samples, 186 (73.2%) amplified the *pfrnr2e2* singly copy gene. Failure in amplification of *pfrnr2e2* was associated with low parasitemia levels (Fig 2A). In samples with parasitemia levels of 3 parasites/ $\mu$ L and above, more than 95% of all samples were amplified successfully. In samples with parasitemia > 100

parasites/ $\mu$ L, the lower limit of detection for PfHRP2-based RDTs, all qPCR reactions were positive for *pfrnr2e2*. Parasitemia levels determined by using the amplification of *pfrnr2e2* correlated closely with parasite densities obtained from *P. falciparum* diagnostic qPCR assays (Fig 2B), this is supported by the findings of the Bland-Altman plot which demonstrates a high order of agreement (Fig 2C). The average ratio of parasite quantification based on diagnostic qPCR assays and qHRP2/3-del assay is 0.8 (95% CI: -1.7-3.3). In summary, the qHRP2/3-del assay amplifies 95 % of samples with parasitemia levels of 3 parasites/ $\mu$ L and above and can be used to reliably quantify parasite levels in field samples.

### **Identification of *pfhrp2* and *pfhrp3* gene deletions using qHRP2/3-del assay**

Next, we wanted to establish the performance of the qHRP2/3-del assay in comparison with the currently used gold standard. Samples with known *pfhrp2/3* deletion status obtained from four different sources were included. Serial dilutions of DNA purified from PfDD2 (*pfhrp2* deletion), PfHB3 (*pfhrp3* deletion) and PfIS (no deletion) served as controls. Samples from CHMI using PfNF54 (no deletion) were added to test the specificity of the qHRP2/3-del assay. Two sample sets genotyped by nested PCR, one from Peru (PE), with a high proportion of *pfhrp2/3* deletions and another one from Tanzania (TZ) dominated by *P. falciparum* strains without deletions were analysed. The qHRP2/3-del assay defines a deletion as failure of amplification of the *pfhrp2/3* genes (Fig 3A, y axis, Cq set to 45) in samples which are positive for the internal control, *pfrnr2e2* (Fig 3A, x axis). Sensitivity is defined as the proportion of correctly identified *pfhrp2/3* deletions, while specificity is the proportion of correctly identified strains without *pfhrp2/3* deletions. All control samples with known deletion status were identified as expected (Fig 3A, first panel). Importantly, the qHRP2/3-del assay correctly identified samples with parasitemia levels ranging from 1-10'0000 parasites/ $\mu$ L, demonstrating the dynamic range of at least 5 logs of this assay. In samples

collected from volunteers that have undergone CHMI with PfNF54 (CHMI, n=38), one sample with a double deletion of the *pfhrp2/3* genes was detected wrongly, resulting in a reduced specificity (Fig 3A, second panel). A high sensitivity was achieved with the Peruvian samples (PE, n=67), *pfhrp2* and *pfhrp3* deletions were detected with sensitivity of 94.4% and 94.9%, respectively (Fig 3A, third panel). The low specificity of 76.9% and 87.5% for *pfhrp2* and *pfhrp3*, respectively, is based on the incorrect detection of deletions in three samples. Among the samples from Tanzania (TZ, n=56), no *pfhrp2/3* deletions were detected by the nested PCR. In contrast, the qHRP2/3-del assay identified three deletions, resulting in a specificity of 93.8% (Fig 3A, fourth panel).

Grouping the samples with missed deletions (reducing the sensitivity) and the false deletions (reducing the specificity) by parasitemia levels revealed a high proportion of false deletions among the samples with the lowest parasitemia levels (Fig 3B). Based on these findings, the inclusion criteria for samples to be analysed by qHRP2/3-del assay was changed. The threshold for the *pfmr2e2* gene amplification was reduced from Cq <40 to Cq <37.5, corresponding to parasitemia levels of 5 parasites/μL. Based on these new inclusion criteria, the qHRP2/3-del assay obtained results from 106 samples out of 127 samples (inclusion rate of 83.5%) (Fig 3C). 12 samples were not amplified by the qHRP2/3-del assay and an additional 9 samples excluded based on the new inclusion criteria. Samples which were not amplified by the qHRP2/3-del assay were mainly ultra-low parasite density samples from Tanzania (11 out of 12). In 6 out of 11 samples (54.5%), the nested PCR had detected *pfhrp2* and *pfhrp3* deletions. In contrast, in the remaining 48 Tanzanian samples not a single deletion had been found. This significantly increased proportion (Fisher exact test,  $p < 0.0001$ ) of deletions detected by nested PCR among samples with ultra-low parasitemia levels is a strong indicator that the gold standard is prone to report incorrect presence of deletions at low parasitemia levels.

In 99 out of 106 samples (93.4%), the *pfhrp2/3* deletion status was identical when compared between qHRP2/3-del assay and nested PCR. This is reflected in the near perfect agreement between these two PCR based diagnostic methods for each of the amplified targets. Cohen's kappa was calculated as 0.89 and 0.91 for *pfhrp2* and *pfhrp3*, respectively. In summary, the qHRP2/3-del assay specificity (94.4% and 96.0% for *pfhrp2* and *pfhrp3*, respectively) and sensitivity (94.2% and 94.6% for *pfhrp2* and *pfhrp3*, respectively) were above 90%. The negative predictive value (NPV) was calculated as 94.5% and 94.1 % and the positive predictive value (PPV) as 94.2% and 96.4%, for *pfhrp2* and *pfhrp3*, respectively (Fig 3D).

### **Multiple strain *P. falciparum* infections are masking *pfhrp2* and *pfhrp3* deletions**

In many malaria endemic regions, particularly in sub-Saharan Africa, infections with multiple strains of *P. falciparum* are common <sup>15</sup>. A blood sample carrying multiple *P. falciparum* strains with and without *pfhrp2/3* deletions will result in failure to detect the deletion by nested PCR if the parasitaemia level of the strain without deletion is sufficiently high for amplification. This limitation leads most likely to an underestimation of the prevalence of *pfhrp2/3* gene deletions in regions with high prevalence of multiple strain infections. We reasoned that the qHRP2/3-del assay could offer a solution by calculating the difference between the Cq values obtained for amplification of *pfhrp2* or *pfhrp3* and *pfmr2e2*. To demonstrate the ability of the qHRP2/3-del assay to correctly identify and quantify “hidden” or “masked” *pfhrp2/3* gene deletions in mixed infections, we first tested defined mixtures of DNA from PfNF54 (no *pfhrp2/3* deletions) and PfDD2 (*pfhrp2* deletion) or PfHB3 (*pfhrp3* deletion) in a range of different ratios. For each combination of strain mixtures, PfDD2/PfNF54 or PfHB3/PfNF54, 10 mixtures were prepared containing varying ratios of strains with and without a *pfhrp2/3* deletion (Fig 4A). The contribution from PfDD2 and PfHB3 strains to these mixtures ranged from 0.1% to 88% and 0.1% to 86%, respectively. In seven mixtures, the



strain with a deletion constituted the minority (with less than 50% abundance) and in three mixtures the majority (with more than 50% abundance). None of these mixtures failed to amplify the *pfhrp2/3* genes, even if the strain carrying the deletion constituted the majority in the mixture. A positive correlation between abundance of isolate carrying a deletion and an increase of  $\Delta Cq$  ( $Cq$  of *pfhrp2* or *pfhrp3* minus  $Cq$  of *pfnr2e2*) is observed (Fig 4B). The qHRP2/3-del assay does not only successfully identify “masked” *pfhrp2/3* deletions but can also discriminate between mixtures where the strain with the deletion constitutes the majority or minority (Fig 4C). A  $\Delta Cq$  cut-off value of 2.0 was chosen to identify “masked” *pfhrp* gene deletions. Applying this cut-off to our sample collections revealed that two isolates each from Tanzania and Peru have high  $\Delta Cq$  values for both *pfhrp* genes indicative of the presence of “masked” *pfhrp2/3* deletions (Fig 4D). Three additional samples from the Peruvian collection had a  $\Delta Cq$  value  $> 2$  for the *pfhrp2* gene only. No  $\Delta Cq$  values above 2 were found in Equatorial Guinean isolates and among samples collected from volunteers undergoing CHMI (Fig 4D). These experiments demonstrate that by calculating the  $\Delta Cq$  values between  $Cq$  for *pfnr2e2* and *pfhrp2* or *pfhrp3*, “masked” deletions can be identified.

## Discussion

*P. falciparum* strains carrying *pfhrp2/3* deletions are an emerging threat to malaria control and elimination programs around the world. Novel analysis tools enabling high-throughput screening of *P. falciparum* populations from the field are needed. The currently published methods, mostly based on nested PCR, have clear limitations in that these methods are extremely time consuming, prone to detection of incorrect deletions at low parasitemia levels and unable to identify “masked” deletions in multiple strain co-infections.

The sensitivity and specificity of the PfHRP2/3-del assay is comparable to the nested PCR, currently the gold standard. However, the novel qHRP2/3-del assay is well suited for high throughput screening of *P. falciparum* isolates with approximately 30 samples analyzed in less than two hours - including DNA extraction and data analysis. Two additional major advantages of the qHRP2/3-del assay are obvious: firstly, the ability to quantify parasitemia levels and therefore include samples based on parasitemia and secondly, to identify “masked” deletions in multiple strain infections.

The identification of *pfhrp2/3* deletions in samples with low parasitemia levels is difficult since the absence of amplification could be due to lack of sufficient template leading to incorrect reports of deletions. The conventional method regards as valid for interpretation all biological samples for which a reference gene, usually *msh2*, is amplified demonstrating the presence of sufficient template in the PCR reaction. The qHRP2/3-del assay uses a better-defined inclusion criterion, based on the Cq value of its internal control. The pre-defined exclusion criteria of all samples that have parasitemia below 5 parasites/ $\mu$ L will improve the quality, reproducibility and comparability of malaria parasite survey data obtained with the qHRP2/3-del assay.

The ability to detect “masked” *pfhrp2/3* deletions is probably the most interesting feature of the

qHRP2/3-del assay, because it will allow studying the epidemiology of *pfhrp2/3* deletions in malaria endemic regions with a high proportion of infections caused by multiplicity of infections, particular sub-Saharan Africa <sup>15</sup>. The qHRP2/3-del assay correctly identified infections that contain two strains, one with a deletion and the other one without a deletion, based on a difference in the Cq values derived from the amplification of the *pfhrp2/3* gene targets and the *pfprn2e2* control. However, currently we cannot exclude that nucleotide sequence variations located in the binding sites of the oligonucleotides used in the PfHRP2/3-del assay could potentially also lead to variation in  $\Delta Cq$  values.

For the first time we report the possible existence of *pfhrp2* and *pfhrp3* deletions in isolates collected in Tanzania. Two isolates had an increased  $\Delta Cq$  value for both *pfhrp* genes. With Kenya [13], the Democratic Republic of Congo [14], Rwanda [15] and Mozambique [16] four out of six direct neighbouring countries have already reported the presence of *pfhrp2/3* deletions, which makes the existence of such strains in Tanzania likely.

The application of our novel PfHRP2/3-del assay in additional studies including a larger sample size will improve our understanding of the relevance of “masked” *pfhrp2* and *pfhrp3* gene deletions and their impact on reliability of malaria RDT test results.

## Conclusion

The qHRP2/3-del assay presented here is suitable for high-throughput screening of *P. falciparum* strains to identify *pfhrp2/3* gene deletions in different malaria endemic settings, including areas with high a proportion of multiple strain co-infections. With growing availability of qPCR instruments in reference laboratories in sub-Saharan countries, this assay could be used as surveillance method to monitor over time the potential expansion of *P. falciparum* strains carrying *pfhrp2* and *pfhrp3* deletions.

## Methods

### ***P. falciparum* Isolates from Tanzania, Equatorial Guinea and Peru**

In this study a total of 205 *P. falciparum* isolates collected from three different malaria endemic regions, East Africa, West-Central Africa and South America were included. The samples from East Africa (n=90) were collected in rural southern Tanzania (TZ) as part of a malaria baseline survey<sup>16</sup>. The West-Central African isolates (n=47) were identified among blood donors living in Malabo, Equatorial-Guinea (EG)<sup>17</sup>. Both samples sets were analyzed locally, at the Bagamoyo branch of the Ifakara Health Institute and the laboratory of the Equatorial Guinea Malaria Vaccine Initiative using harmonized protocols. Briefly, genomic DNA was isolated either from 6 circles with 2mm diameter of dried blood spots (Tanzania) or 180 µL whole blood (Equatorial Guinea) using the Quick-DNA Miniprep kits (Zymo Research, Irvine, USA). *P. falciparum* was identified and quantified using published qPCR protocols based on varATS<sup>18</sup>. Extracted DNA (n=68) from Peruvian isolates (PE), collected between 2008-2009 and 2015-2016 around Iquitos city, was shipped to the Swiss Tropical and Public Health Institute for *pfhrp2/3* characterization by qHRP2/3-del assay.

### **Additional Parasite Isolates and Laboratory Strains**

Forty-nine PfNF54 isolates from Controlled Human Malaria Infections (CHMI) conducted in Bagamoyo, Tanzania (ClinicalTrials.gov: NCT02613520<sup>19</sup>) as well as genomic DNA isolated from 8 laboratory strains with know *pfhrp2/3* deletion status (Pf3D7, Pf7G8, PfDD2, PfHB3, PfNF135.C10, PfNF166.C8, PfNF54 and PfFC27) were used as controls. The 1st WHO International Standard for *Plasmodium falciparum* DNA Nucleic Acid Amplification Techniques (NIBSC code: 04/176, herein referred to as PfIS) was used to assess the performance of the

qHRP2/3-del assay. Non-falciparum *Plasmodium* species, including *P. malariae* (Pm), *P. ovale curtisi* (Poc), *P. ovale wallikeri* (Pow), *P. vivax* (Pv) and *P. knowlesi* (Pk) and an additional 28 samples from malaria negative individuals living in Tanzania were used to assess specificity of the assay.

### **Detection of the *pfhrp2* and *pfhrp3* genes by conventional nested PCR**

*P. falciparum* positive samples collected in Tanzania were selected for detection of *pfhrp2* and *pfhrp3* genes by nested PCR. As a reference gene, the *msp2* gene was amplified using a previously described protocol <sup>20</sup>. All isolates with successful *msp2* amplification were analyzed for the presence of *pfhrp2* and *pfhrp3* genes using primers spanning exon 1, the intron, and exon 2 <sup>21</sup>. All PCR products were separated and visualized on a 2% agarose gel. Cultured parasite isolate PfDD2 (*pfhrp2* deletion) was used as a control for all nested PCR experiments on *pfhrp2* while PfHB3 (*pfhrp3* deletion) was used as a control for all nested PCR experiments on *pfhrp3*. PfNF54 (no *pfhrp2/3* deletion) was used as a positive control for both *pfhrp* genes. *Pfhrp2/3* deletion status of the Peruvian *P. falciparum* isolates was analyzed previously following the procedures described in <sup>9</sup> and the results were shared to be used for the evaluation of the qHRP2/3-del assay.

### **Design of qHRP2/3-del assay**

Published *pfhrp2/3* primer sequences for conventional PCR were adapted to the qPCR platform using EvaGreen® qPCR Mix (Solis BioDyne, Tartu, Estonia). The primers were tested with different DNA concentrations extracted from PfNF54, PfDD2 and PfHB3 strains. The best performing primer pairs, in terms of specificity and sensitivity, were then used in combination with newly designed TaqMan® hydrolysis probes. The *pfhrp2/3* oligo sequences were systematically optimized using the trial-and-error approach. As the internal control of the qHRP2/3-del assay we

amplify a *P. falciparum* specific 107 bp long sequence of the ribonucleotide reductase R2\_e2 (*pfrnr2e2*), a distantly related paralog of the canonical eukaryotic small subunit ribonucleotide reductase R2, that is unique to apicomplexan species <sup>14</sup>. Genomic sequences for *pfrnr2e2* (PF3D7\_1015800), *pfhrp2* (PF3D7\_0831800) and *pfhrp3* (PF3D7\_1372200) of Pf3D7 strain were obtained from PlasmoDB. A *pfhrp2/3* sequence alignment including five reference strains from West-Africa (Pf3D7, PfNF54), Guinea (PfNF166.C8), Brazil (Pf7G8) and Cambodia (PfNF135.C10) revealed no SNPs in oligo binding regions suggesting a high degree of conservation within the target region of the *pfhrp2/3* genes (Supplementary file 2 and 3). The Geneious version 8.1.9 software (Biomatters Ltd, Auckland, New Zealand) was used for sequence alignments and oligo designs. Relevant information concerning the oligos used in the qHRP2/3-del assay is summarized in Table 1.

### **Sample analysis with qHRP2/3-del assay**

Amplification and qPCR measurements were performed using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). The thermal profile used for qHRP2/3-del assay is as follows: Taq polymerase activation for 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 35 s at 57.5 °C. 2 µL DNA was added to 8 µL reaction master mix containing 1x Luna Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, USA) and 1x qHRP2/3-del Primer Mix (Table 1). All qPCR assays were run in triplicates with appropriate controls including Non-Template Control and DNA from PfDD2, PfHB3 and PfNF54 as controls for the *pfhrp2/3* deletion status.

## Data management and statistical analysis

*Preliminary analysis of qPCR data.* Cq values were obtained from the Bio-Rad CFX96 Manager 3.1 software (Bio-Rad Laboratories, California, USA) after setting the threshold manually. Cq values were transferred and linked to the samples' metadata using a custom-designed database for storage and analysis of qPCR data. Only samples with a  $Cq \leq 40.0$  for the internal control, *pfmr2e2*, were considered eligible for analysis of *pfhrp2/3* deletion status.  $\Delta Cq$  were calculated by subtraction of *pfmr2e2* Cq values from *pfhrp2* or *pfhrp3* Cq values.

*Analytical performance of qHRP2/3-del assay and quantification of parasitemia.* Based on the PfIS a serial dilution ranging from 0.01-10'000 parasites/ $\mu$ L was prepared and used to assess the performance of the qHRP2/3-del assay. The slope, y-intercept, qPCR efficiency and  $R^2$  was established for each target. The Limit of Detection (LOD) was defined as the lowest PfIS parasitemia with a positive amplification in 4 out of 6 replicates. Parasitemia was estimated using linear regression derived from serial dilution of the PfIS and the *pfmr2e2* target which serves as the internal control of the qHRP2/3-del assay.

*Graphical representation and statistical analysis.* We used R version 3.5.1 for creating ggplot2-based graphs using the packages ggpubr, gridextra and scales. The Diagnostic test evaluation calculator (freely available at [https://www.medcalc.org/calc/diagnostic\\_test.php](https://www.medcalc.org/calc/diagnostic_test.php)) was used for analytical validation of qHRP2/3-del assay performance. Cohen's kappa including 95% confidence intervals, providing a measure of agreement, was calculated using STATA version 12.0 software (Stata Corp LP; College Station, Texas, USA). P values  $< 0.05$  were considered as significant for all statistical analysis.



**Table 1. Oligonucleotide sequences used for qHRP2/3-del assay**

Target gene	Size	Oligo name	Oligo sequence [5'to 3']	Fluorophores	Conc. in 5x PrimerMix <sup>a</sup>
<i>pfhrp2e2</i>	107 bp	IC-PfRNR2E2 fwd	AGTATCCAAAACACTATAATTCCAAGTAC	-	1.5 µM
(PF3D7_1015800)		IC- PfRNR2E2 rev	ATTTCTCCTTCTTAACAGTTTCTTCC	-	1.5 µM
		IC-PfRNR2E2 Cy5	CCTTTTAGTTGGCCCGAATTACAA	Cy5-BHQ2	1.125 µM
<i>pfhrp2</i>	286 bp	PfHRP2 fwd	GTATTATCCGCTGCCGTTTTTGCC <sup>b</sup>	-	1.5 µM
(PF3D7_0831800)		PfHRP2 rev	TCTACATGTGCTTGAGTTTCG <sup>b</sup>	-	1.5 µM
		PfHRP2 TxRd	TTCCGCATTTAATAATAACTTGTGTAGC	TexasRed-BHQ2	0.375 µM
<i>pfhrp3</i>	289 bp	PfHRP3 fwd	ATATTATCCGCTGCCGTTTTTGCT	-	1.5 µM
(PF3D7_1372200)		PfHRP3 rev	CCTGCATGTGCTTGACTTTCGT	-	1.5 µM
		PfHRP3 YY	CTCCGAATTTAACAATAACTTGTTTAGC	YakimaYellow-BHQ2	0.75 µM

<sup>a</sup> All oligonucleotides are premixed as a 5x primer mix

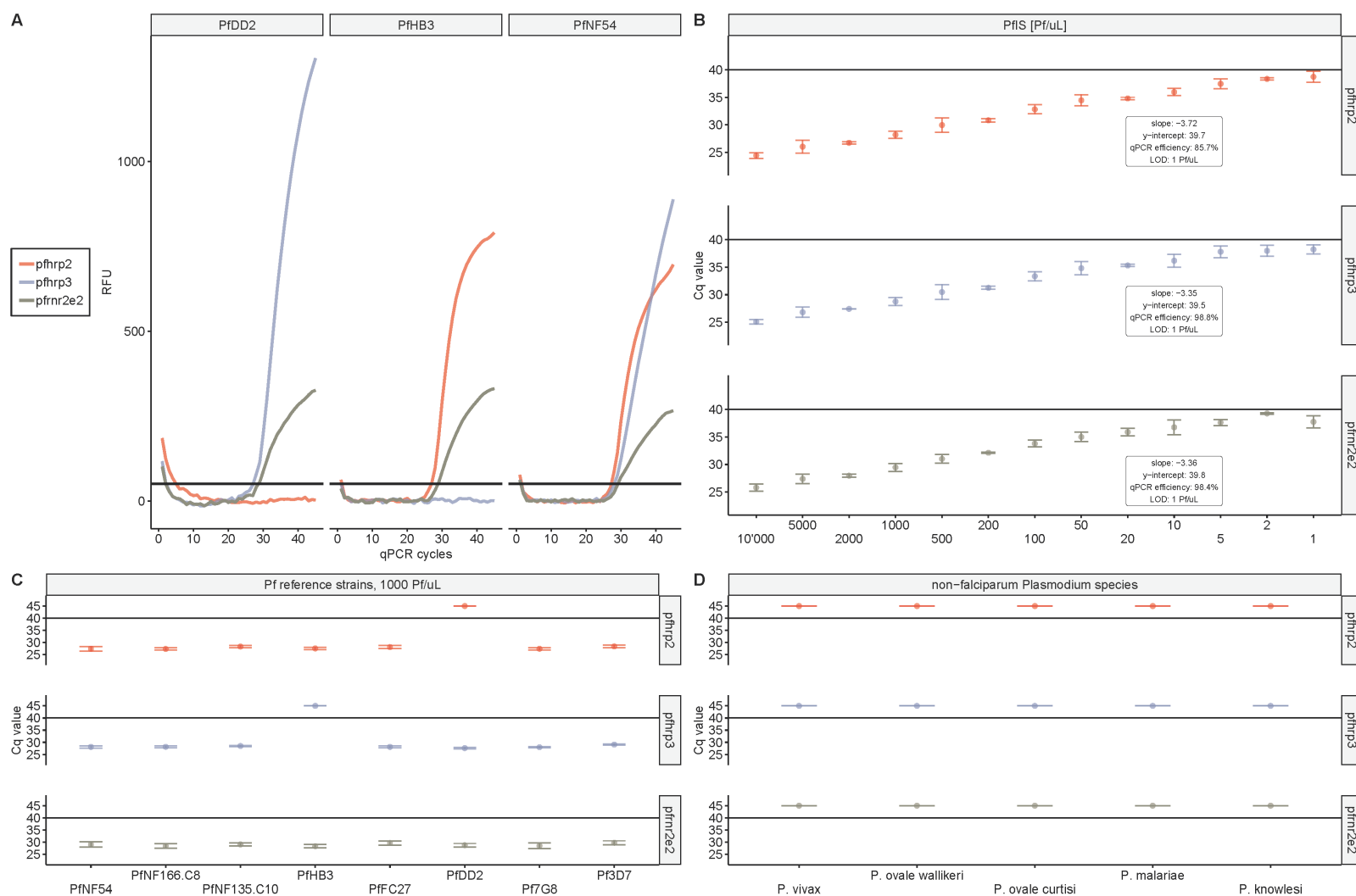
<sup>b</sup> oligonucleotide sequences obtained from Abdallah *et al.* <sup>21</sup>

**Table 2. Field samples used for evaluation of qHRP2/3-del assay**

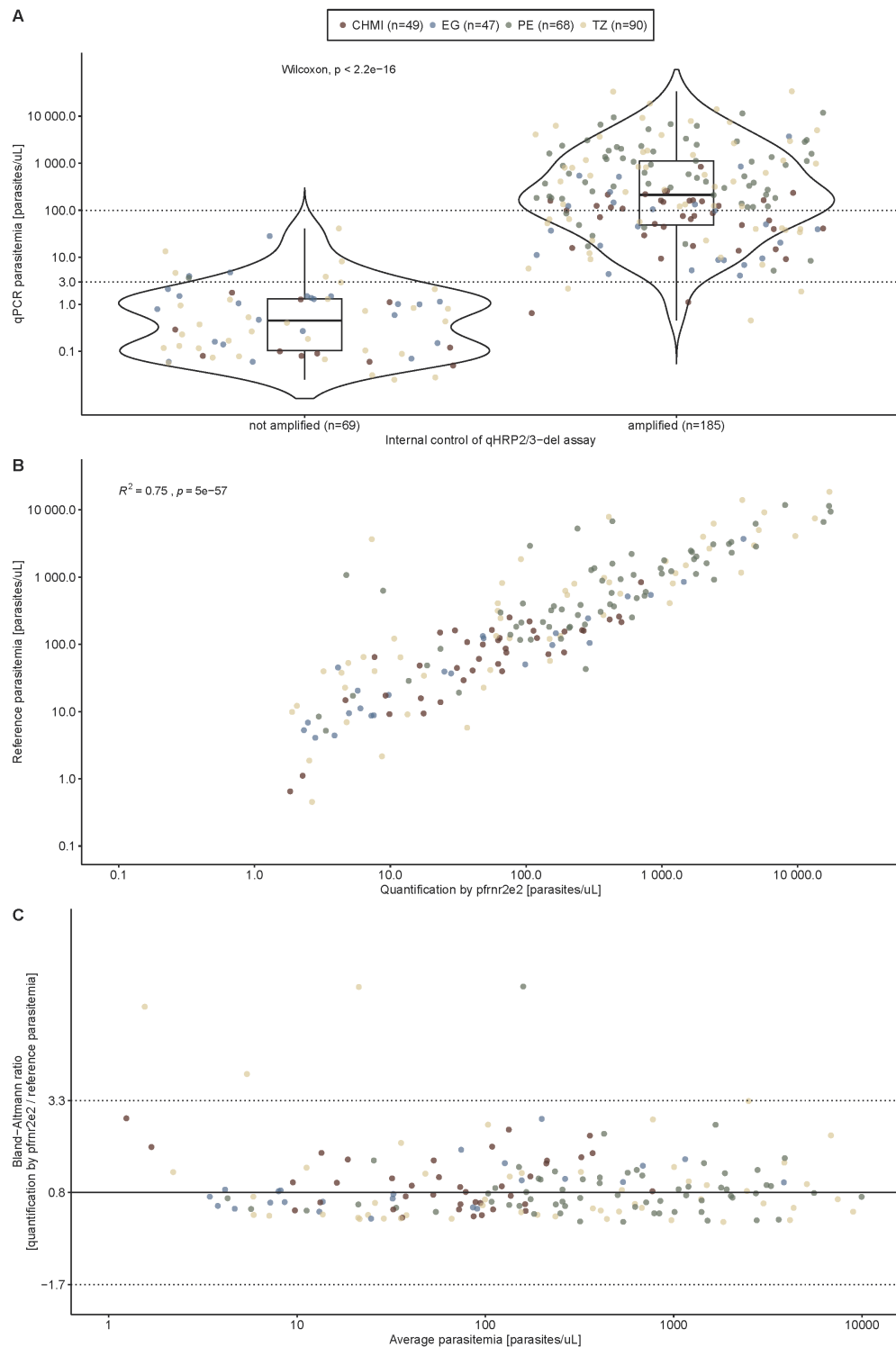
Sample set	Description of sample set	Number of <i>P. falciparum</i> positive samples <sup>b</sup>	Parasitemia in parasites/µL (Median/IQR)	Amplification rate by qHRP2/3-del assay
CHMI <sup>a</sup>	CHMI in TZ with PfNF54 strain (no deletion)	49	51.1 (1.5-152.5)	78%
PE	Peruvian samples around Iquitos city. High proportion of <i>pfhrp2/3</i> deletions	68	592.4 (186.7-1982.0)	99%
EG	Blood donors with asymptomatic malaria infection living on Bioko Island, Equatorial Guinea	47	4.8 (1.0-45.3)	51%
TZ	Sampling of symptomatic volunteers at two health facilities in Southern Tanzania	90	38.8 (0.7-808.6)	62%
<b>Combined</b>		<b>254</b>	<b>75.7 (2.2-571.6)</b>	<b>73%</b>

<sup>a</sup> Controlled Human Malaria Infection

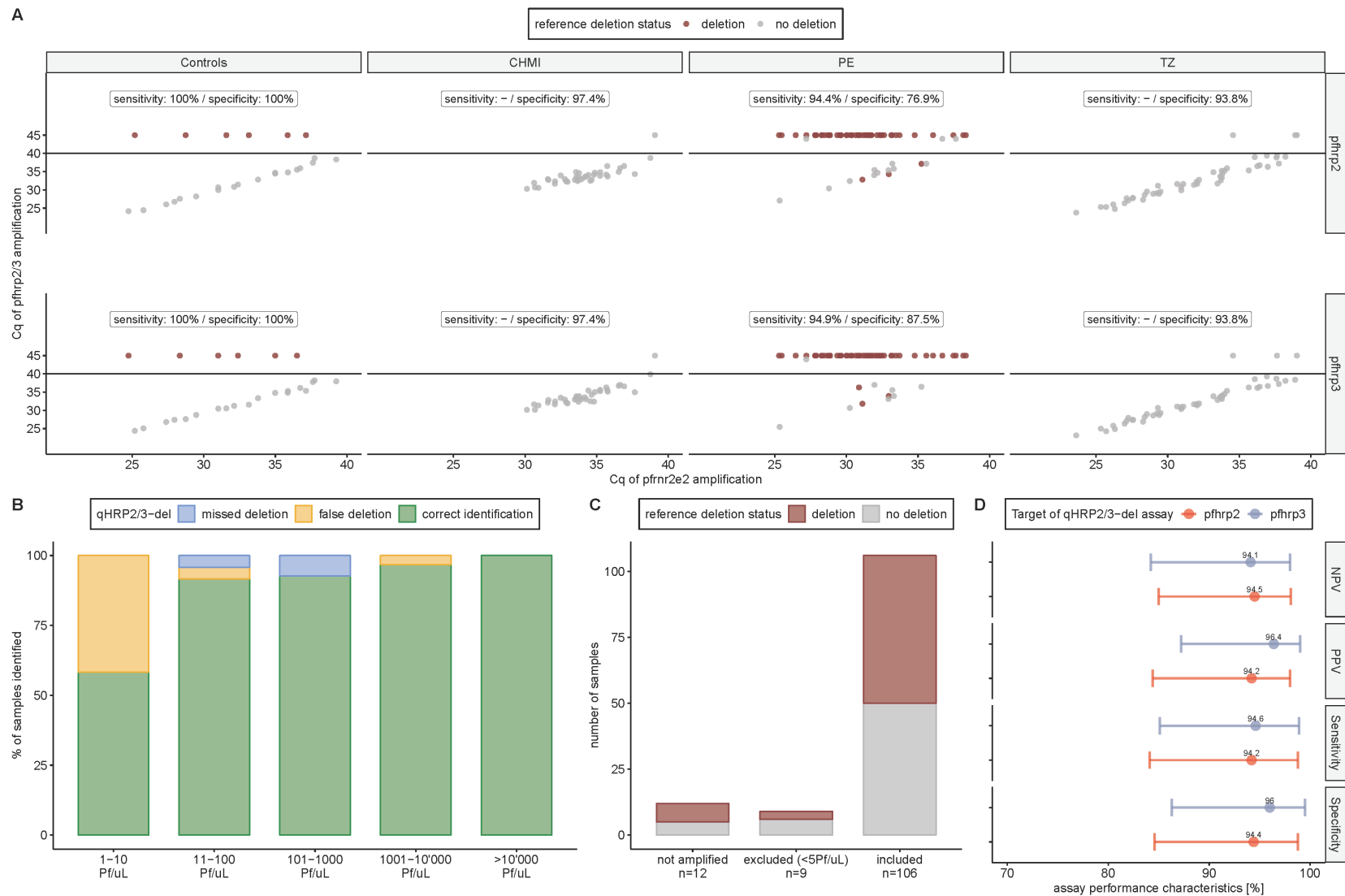
<sup>b</sup> all confirmed by diagnostic qPCR assays



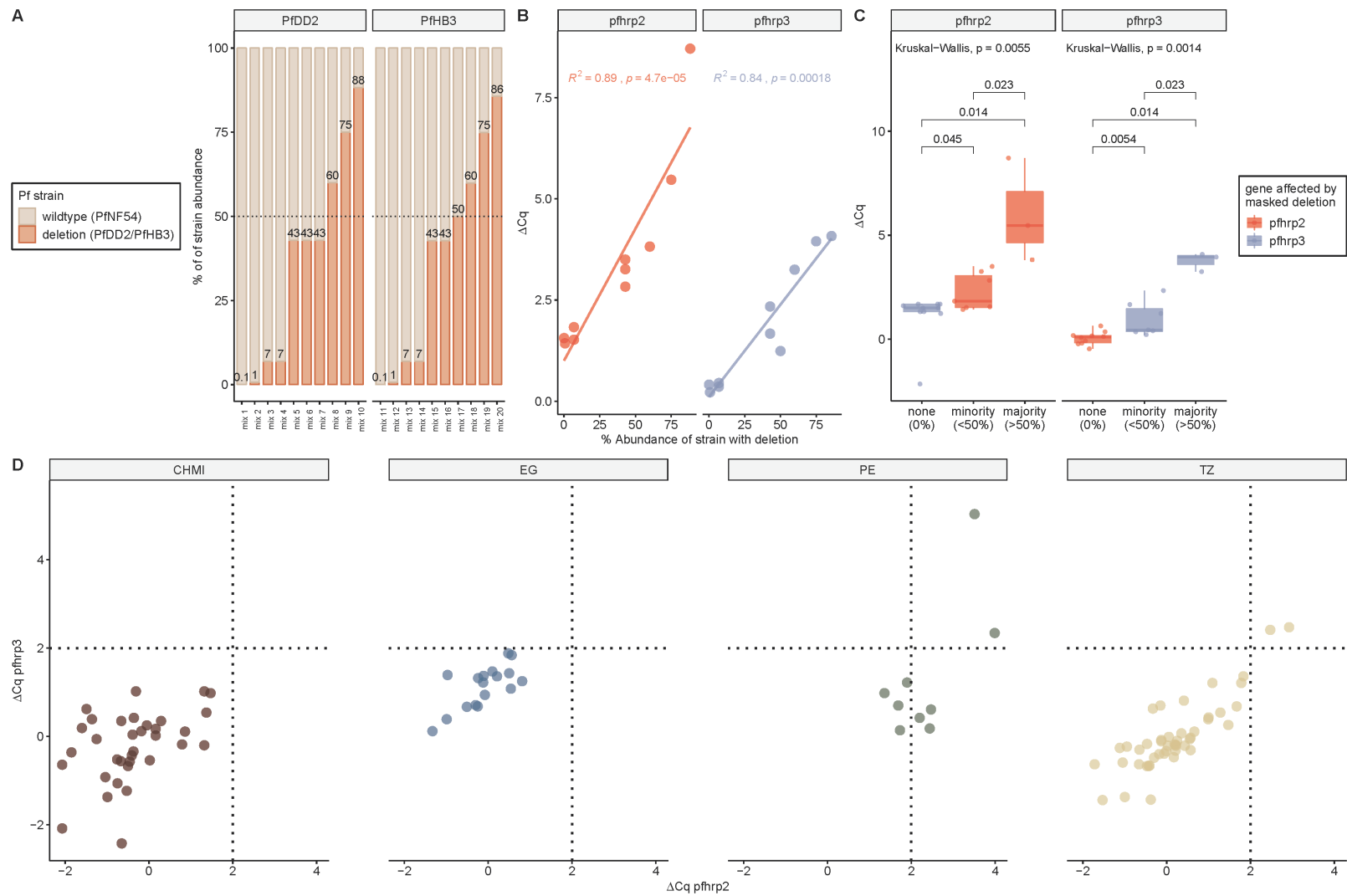
**Figure 1. Multiplex detection of *pfhrp2* and *pfhrp3* genes using the qHRP2/3-del assay.** (A) The qHRP2/3-del assay amplifies *pfhrp2*, *pfhrp3* and *pfmr2e2* target sequences in a multiplex qPCR reaction and correctly identifies strains carrying either a *pfhrp2* deletion (PfDD2), a *pfhrp3* deletion (PfHB3) or no deletion (PfNF54). (B) Performance characteristic of each individual amplification assay, run within the multiplex qHRP2/3-del assay, is shown. Correlation with high linearity between serially diluted WHO international standard for *P. falciparum* NATs (PfIS) and Cq values was obtained and used to calculate the qPCR efficiency. Cq values above 40 (black line) are considered negative. (C) DNA extracted from cultured parasites diluted to 1000 parasites/ $\mu$ L was used to demonstrate performance of the qHRP2/3-del assay in strains representing a global collection of *P. falciparum* parasites. (D) qHRP2/3-del assay specificity for *P. falciparum* was tested using DNA derived from non-*falciparum* *Plasmodium* species.



**Figure 2. Detection and quantification of field samples using qHRP2/3-del assay.** (A) Amplification rate of *pfmr2e2* target, the internal control of qHRP2/3-del assay and association with parasitemia levels. Wilcoxon-Mann-Whitney test was used for comparison of parasitemia between groups. The dashed line at 3 parasites/ $\mu$ L represents the parasitemia at which more than 95% of the samples were amplified, while the dashed line at 100 parasites/ $\mu$ L represents the LOD of RDTs at which all samples are successfully amplified. (B) Correlation of parasitemia levels, obtained from diagnostic qPCR assays, and parasitemia, derived from the internal control of qHRP2/3-del assay, is shown. The color represents the different sample sets and  $R^2$  the Spearman's rank correlation coefficient. (C) Bland-Altman plot of average parasitemia (x-axis) and ratio of parasitemia levels calculated between internal control of qHRP2/3-del assay and diagnostic qPCR assays (y-axis). Average ratio (black line) and 95 % limits of agreement (dashed line) are depicted.



**Figure 3. Diagnostic performance of qHRP2/3-del assay.** (A) Samples with Cq values for *pfrp2* and *pfrp3* amplification > 40 (shown on y-axis, black line indicates cut-off) are considered to carry a *pfrp2/3* deletion. Reference deletion status, based on nested PCR, is color coded (red = deletion, grey = no deletion). (B) Proportion of correctly and incorrectly identified *pfrp2/3* deletion status grouped by parasitemia. (C) Number of samples included for analysis by qHRP2/3-del assay (n=106), excluded due to ultra-low parasitaemia (n=9) and not amplified (n=12). (D) Analytical validation of qHRP2/3-del assay performance was assessed by comparing it to nested PCR (gold standard). Standard parameters such as sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) including their 95% confidence intervals are shown.



**Figure 4. Identification of masked *pfhrp2/3* deletions in multiple strain infections.** (A) Mixtures containing two strains, one with a *pfhrp* deletion (PfDD2 or PfHB3) and no deletion (PfNF54), were generated. (B) Correlation between abundance of strain carrying deletion and  $\Delta Cq$  is shown for both targets, *pfhrp2* (red) and *pfhrp3* (blue). (C) The  $\Delta Cq$  approach distinguishes between strain mixtures not carrying deletions, mixtures with minority abundance as well as majority abundance of strains with deletions. Statistical comparison was performed using the Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney for pairwise comparisons. (D) The  $\Delta Cq$  approach of the qHRP2/3-del assay was applied to four sample collections to identify “masked” *pfhrp2/3* deletions. The control group, based on samples from CHMI, did not reveal isolates with increased  $\Delta Cq$  values. The dashed lines represent the  $\Delta Cq$  cut-off values for *pfhrp2* (x-axis) and *pfhrp3* (y-axis).

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## Author contributions statement

Study concept and design: TS, ACD, CD. Acquisition of data: TS, ACD, MF, EG, SMM. Analyses and interpretation of data: TS, ACD, DG, PM, CD. Drafting the manuscript: TS, ACD, CD. Technical or material support: KM, JCS, DG, PM, KT, SA, SLH, MT. Sample collection and enrollment of patients: MGM, SAJ, PPC, JRB, PM, KT. Study supervision: CD. All authors read and approved the final manuscript.

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## **Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

## **Supplementary information**

Supplementary file 1. Sequence homology of *pfhrp2* and *pfhrp3* including oligo binding sites of qHRP2/3-del assay.

Supplementary file 2. Sequence alignment of five reference strains from West-Africa (Pf3D7, PfNF54), Guinea (PfNF166.C8), Brazil (Pf7G8) and Cambodia (PfNF135) for *pfhrp2* gene (first 360 bp).

Supplementary file 3. Sequence alignment of five reference strains from West-Africa (Pf3D7, PfNF54), Guinea (PfNF166.C8), Brazil (Pf7G8) and Cambodia (PfNF135) for *pfhrp3* gene (first 360 bp).

## **Competing interests**

The authors declare no competing interests.

## **Ethical approval and informed consent**

The samples analyzed in this study were collected in different studies. All studies were approved by the appropriate institutions and informed consent was obtained from all participants. The CHMI samples were collected during a clinical study, registered at Clinical Trials.gov (NCT02613520), and conducted under a U.S. FDA IND application. The study was performed in accordance with Good Clinical Practices. All samples analyzed in this publication were obtained according to the approved study protocol. The protocol was approved by the institutional review boards of the Ifakara Health Institute (IHI/IRB/No: 32-2015), and the

National Institute for Medical Research Tanzania (NIMR) (NIMR/HQ/R.8a/Vol.IX/2049), by the Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland (Ref. No. 15/104), and by the Tanzania Food and Drug Authority (Auth. No. TZ15CT013). For the Tanzanian sample collection ethics approval for the study was granted by the institutional review boards of Ifakara Health Institute (IHI/IRB/No: 18-2015) and by NIMR (NIMR/HQ/R.8a/Vol.IX/2015). For the sample collection from Equatorial Guinea approval was given by the Ministry of Health and Social Welfare. The collection, transport and storage of the blood samples from Peru was approved by the Human Ethics Committee from Universidad Peruana Cayetano Heredia (UPCH 52707 & 59751).

## Abbreviations

*pfhrp2* (parasite-derived histidine-rich protein 2), *pfhrp3* (parasite-derived histidine-rich protein 3), RDT (rapid diagnostic test), qPCR (quantitative polymerase chain reaction), qHRP2/3-del (qPCR assay for quantitative detection of *pfhrp2* and *pfhrp3* deletions), PfIS (1st WHO International Standard for *Plasmodium falciparum* DNA Nucleic Acid Amplification Techniques), NPV (negative predictive value), PPV (positive predictive value), CHMI (Controlled human malaria infection), EG (Equatorial Guinea), PE (Peru), TZ (Tanzania)



**Supplementary file 1.** Sequence alignment (first 350 bp) of pfhrp2 and pfhrp3 genes including intron sequence (grey). qHRP2/3-del primer (black) and probe (green) binding regions are highlighted. Nucleotide sequence differences in oligo binding regions between *pfhrp2* and *pfhrp3* are indicated in red.

pfhrp2	1	ATGGTTTCCTTCTCAAAAAATAAA	<u>GTATTATCCGCTGCCGTTTTTGCC</u>	TC	50
pfhrp3	1	ATGGTTTCCTTCTCAAAAAATAAA	<u>ATATTATCCGCTGCCGTTTTTGCT</u>	TC	50
pfhrp2	51	CGTACTTTTGTTAGATAAC	CGTAAGCATTTTAATTGCAAATAGAAATAAAAT		100
pfhrp3	51	CGTACTTTTGTTAGATAAC	CGTAAGTATTTTAATTGCAAATATAAATAAAAT		100
pfhrp2	101	ATATCATTCAATTATAAAAAATACATATAATAATAATATATATATATATAT			150
pfhrp3	101	ATAACACTTACTTATAAAAAATACATATAATAATATTATATATATATATAT			150
pfhrp2	151	ATATATTTATATATAT-----TGTATATATATAAATTTTTTTCATTTTTAAAT			195
pfhrp3	151	ATATATATATATATATATATATATATATGTACATTTTACATTTTTTAAAT			200
pfhrp2	196	GCTTTTTTATTTTTATATAGAATAA	<u>TCCGCATTTAATAATAACTTGTGT</u>		245
pfhrp3	201	GATTTTTCATTTT--TATAGAATAA	<u>CTCCGAATTTAACAAATAACTTGT</u>		248
pfhrp2	246	AGCAAAAATGCAAAAGGACTTAATTTAAATAAGAGATTATTAC	<u>ACGAAAC</u>		295
pfhrp3	249	AGCAAAAATGCAAAAGGACTTAATTCAAATAAGAGATTATTAC	<u>ACGAAAG</u>		298
pfhrp2	296	TCAAGCACATG	<u>TAGA</u>	TGATGCCCATCATGCTCATCATGTAGCCGATGCC	345
pfhrp3	299	TCAAGCACATG	<u>CAGG</u>	TGATGCCCATCATGCACATCATGTAGCTGATGCC	348

**Supplementary file 2.** Sequence alignment (first 360 bp) of *pfhrp2* for five reference strains from Africa (3D7, NF54, NF166.C8), Brazil (7G8) and Cambodia (NF135.C10). The oligo binding sites are highlighted in grey.

NF135	ATGGTTTCCTTCTCAAAAAATAAAGTATTATCCGCTGCCGTTTTTGCCCTCCGTACTTTTG	60
3D7	ATGGTTTCCTTCTCAAAAAATAAAGTATTATCCGCTGCCGTTTTTGCCCTCCGTACTTTTG	60
7G8	ATGGTTTCCTTCTCAAAAAATAAAGTATTATCCGCTGCCGTTTTTGCCCTCCGTACTTTTG	60
NF54	ATGGTTTCCTTCTCAAAAAATAAAGTATTATCCGCTGCCGTTTTTGCCCTCCGTACTTTTG	60
NF166	ATGGTTTCCTTCTCAAAAAATAAAGTATTATCCGCTGCCGTTTTTGCCCTCCGTACTTTTG	60
*****		
NF135	TTAGATAACGTAAGCATTTTAATTGCAAATAGAAATAAATATATCATTCAATTATAAAAA	120
3D7	TTAGATAACGTAAGCATTTTAATTGCAAATAGAAATAAATATATCATTCAATTATAAAAA	120
7G8	TTAGATAACGTAAGCATTTTAATTGCAAATAGAAATAAATATATCATTCAATTATAAAAA	120
NF54	TTAGATAACGTAAGCATTTTAATTGCAAATAGAAATAAATATATCATTCAATTATAAAAA	120
NF166	TTAGATAACGTAAGCATTTTAATTGCAAATAGAAATAAATATATCATTCAATTATAAAAA	120
*****		
NF135	TACATATAATAATAATATATATATATATATATATATTTATATATTGTATATATATAAATT	180
3D7	TACATATAATAATAATATATATATATATATATATATTTATATATTGTATATATATAAATT	180
7G8	TACATATAATAATAATATATATATATATATATATATTTATATATTGTATATATATAAATT	180
NF54	TACATATAATAATAATATATATATATATATATATTTATATATTGTATATATATAAATT	180
NF166	TACATATAATAATAATATATATATATATATATATTTATATATTGTATATATATAAATT	180
*****		
NF135	TTTTCATTTTTAAATGCTTTTTTATTTTTATATAGAATAATCCGCATTTAATAATAACT	240
3D7	TTTTCATTTTTAAATGCTTTTTTATTTTTATATAGAATAATCCGCATTTAATAATAACT	240
7G8	TTTTCATTTTTAAATGCTTTTTTATTTTTATATAGAATAATCCGCATTTAATAATAACT	240
NF54	TTTTCATTTTTAAATGCTTTTTTATTTTTATATAGAATAATCCGCATTTAATAATAACT	240
NF166	TTTTCATTTTTAAATGCTTTTTTATTTTTATATAGAATAATCCGCATTTAATAATAACT	240
*****		
NF135	TGTGTAGCAAAAATGCAAAGGACTTAATTTAAATAAGAGATTATTACACGAAACTCAAG	300
3D7	TGTGTAGCAAAAATGCAAAGGACTTAATTTAAATAAGAGATTATTACACGAAACTCAAG	300
7G8	TGTGTAGCAAAAATGCAAAGGACTTAATTTAAATAAGAGATTATTACACGAAACTCAAG	300
NF54	TGTGTAGCAAAAATGCAAAGGACTTAATTTAAATAAGAGATTATTACACGAAACTCAAG	300
NF166	TGTGTAGCAAAAATGCAAAGGACTTAATTTAAATAAGAGATTATTACACGAAACTCAAG	300
*****		
NF135	CACATGTAGATGATGCCCATCATGCTCATCATGTAGCCGATGCCCATCATGCTCATCATG	360
3D7	CACATGTAGATGATGCCCATCATGCTCATCATGTAGCCGATGCCCATCATGCTCATCATG	360
7G8	CACATGTAGATGATGCCCATCATGCTCATCATGTAGCCGATGCCCATCATGCTCATCATG	360
NF54	CACATGTAGATGATGCCCATCATGCTCATCATGTAGCCGATGCCCATCATGCTCATCATG	360
NF166	CACATGTAGATGATGCCCATCATGCTCATCATGTAGCCGATGCCCATCATGCTCATCATG	360
*****		

**Supplementary file 3.** Sequence alignment (first 360 bp) of *pfhrp3* for five reference strains from Africa (3D7, NF54, NF166.C8), Brazil (7G8) and Cambodia (NF135.C10). The oligo binding sites are highlighted in grey.

NF166	ATGGTTTCCTTCTCAAAAAATAAAATATTATCCGCTGCCGTTTTTGCTTCCGTACTTTTG	60
NF135	ATGGTTTCCTTCTCAAAAAATAAAATATTATCCGCTGCCGTTTTTGCTTCCGTACTTTTG	60
3D7	ATGGTTTCCTTCTCAAAAAATAAAATATTATCCGCTGCCGTTTTTGCTTCCGTACTTTTG	60
7G8	ATGGTTTCCTTCTCAAAAAATAAAATATTATCCGCTGCCGTTTTTGCTTCCGTACTTTTG	60
NF54	ATGGTTTCCTTCTCAAAAAATAAAATATTATCCGCTGCCGTTTTTGCTTCCGTACTTTTG	60
*****		
NF166	TTAGATAACGTAAGTATTTTAATTGCAAAATATAAAATAAATAACACTTACTTATAAAAA	120
NF135	TTAGATAACGTAAGTATTTTAATTGCAAAATATAAAATAAATAACACTTACTTATAAAAA	120
3D7	TTAGATAACGTAAGTATTTTAATTGCAAAATATAAAATAAATAACACTTACTTATAAAAA	120
7G8	TTAGATAACGTAAGTATTTTAATTGCAAAATATAAAATAAATAACACTTACTTATAAAAA	120
NF54	TTAGATAACGTAAGTATTTTAATTGCAAAATATAAAATAAATAACACTTACTTATAAAAA	120
*****		
NF166	TACATATAATAATATTATATATATATATATATATATATATATATATATATATATATATGT	180
NF135	TACATATAATAATATTATATATATATATATATATATATATATATATATATATATATATGT	180
3D7	TACATATAATAATATTATATATATATATATATATATATATATATATATATATATATATGT	180
7G8	TACATATAATAATATTATATATATATATATATATATATATATATATATATATATATATGT	180
NF54	TACATATAATAATATTATATATATATATATATATATATATATATATATATATATATATGT	180
*****		
NF166	ACATTTTACATTTTAAATGATTTTTCATTTTATAGAATAACTCCGAATTTAACAATA	240
NF135	ACATTTTACATTTTAAATGATTTTTCATTTTATAGAATAACTCCGAATTTAACAATA	240
3D7	ACATTTTACATTTTAAATGATTTTTCATTTTATAGAATAACTCCGAATTTAACAATA	240
7G8	ACATTTTACATTTTAAATGATTTTTCATTTTATAGAATAACTCCGAATTTAACAATA	240
NF54	ACATTTTACATTTTAAATGATTTTTCATTTTATAGAATAACTCCGAATTTAACAATA	240
*****		
NF166	ACTTGTTTAGCAAAAATGCAAAAGGACTTAATTCAAATAAGAGATTATTACACGAAAGTC	300
NF135	ACTTGTTTAGCAAAAATGCAAAAGGACTTAATTCAAATAAGAGATTATTACACGAAAGTC	300
3D7	ACTTGTTTAGCAAAAATGCAAAAGGACTTAATTCAAATAAGAGATTATTACACGAAAGTC	300
7G8	ACTTGTTTAGCAAAAATGCAAAAGGACTTAATTCAAATAAGAGATTATTACACGAAAGTC	300
NF54	ACTTGTTTAGCAAAAATGCAAAAGGACTTAATTCAAATAAGAGATTATTACACGAAAGTC	300
*****		
NF166	AAGCACATGCAGGTGATGCCCATCATGCACATCATGTAGCTGATGCCCATCATGCACATC	360
NF135	AAGCACATGCAGGTGATGCCCATCATGCACATCATGTAGCTGATGCCCATCATGCACATC	360
3D7	AAGCACATGCAGGTGATGCCCATCATGCACATCATGTAGCTGATGCCCATCATGCACATC	360
7G8	AAGCACATGCAGGTGATGCCCATCATGCACATCATGTAGCTGATGCCCATCATGCACATC	360
NF54	AAGCACATGCAGGTGATGCCCATCATGCACATCATGTAGCTGATGCCCATCATGCACATC	360
*****		

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# High Throughput Extraction and Analysis of Nucleic Acids from Rapid Diagnostic Tests for Molecular Surveillance of Malaria

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## Abstract

The use of malaria rapid diagnostic tests (RDTs) as a source for nucleic acids that can be detected by nucleic acid amplification techniques (NATs) would have several advantages, including the small amount of blood and cost-effectiveness regarding sample collection as well as simplified storage and shipping conditions under room temperature (RT). We developed systematically and evaluated extensively a procedure to extract total nucleic acids from used malaria RDTs. The ENAR (Extraction of Nucleic Acids from RDTs) protocol, co-extracts DNA and RNA from small volumes of dried blood and allows the detection and quantification of *P. falciparum* parasites from asymptomatic patients with parasite densities as low as 1 parasite/ $\mu$ L blood using reverse transcription quantitative PCR. The ENAR protocol was evaluated using controlled human malaria infections as a standardized platform which allowed us to conclude that nucleic acids can be reliably recovered and amplified from RDTs, even after long-term storage at RT. Our approach is supported by custom-made software solutions allowing to analyse thousands of RDTs in a standardized and high-throughput manner.

**Keywords:** Nucleic Acid Extraction, Molecular Malaria Surveillance, Rapid Diagnostic Test (RDT), Quantitative Polymerase Chain Reaction (qPCR)

**Abbreviations:** RDT (rapid diagnostic test), DBS (dried blood spot), ENAR (extraction of nucleic acids from RDT), CHMI (controlled human malaria infection), NA (nucleic acids), NAT (nucleic acid amplification technique), PfIS (WHO International standard for *P. falciparum* NAT), LOD (limit of detection), RT (room temperature)



## Introduction

Malaria is a vector-borne, global public health problem caused by apicomplexan parasites of the genus *Plasmodium*. WHO published that in 2017, 219 million cases in 87 countries occurred with an estimated number of 435 000 malaria deaths (1). Currently used malaria control strategies include vector control measures like sleeping under insecticide-treated mosquito nets and indoor residual spraying with insecticides as well as access to timely malaria diagnostic testing and prompt treatment (1). In Sub-Saharan Africa, the region which carries the highest burden of malaria, the majority of countries collect their malaria surveillance data through national health information systems (1). The parasite rate, the proportion of population found to carry asexual blood-stage parasites in their peripheral blood, is the most commonly recorded measure of malaria prevalence, reflecting closely the malaria transmission intensity (2, 3).

Malaria rapid diagnostic tests (RDTs) have become the most widely used method to measure parasite rates of asexual blood stage parasitaemia in endemic countries. In Sub-Saharan Africa, RDTs have almost completely replaced light microscopy for malaria diagnosis, with an estimated 75% of malaria tests conducted using RDTs in 2017 (1). This is the consequence of the relatively low costs, rapid generation of diagnostic results, wide availability and simplicity in use. In 2017 alone, 245 million RDTs were distributed worldwide (1). Technically, RDTs are based on a lateral flow device which detects circulating malaria antigens using antibodies in 5 to 20  $\mu\text{L}$  of capillary blood (4). The vast majority of antibodies in use recognise the parasite-derived histidine-rich protein 2 (PfHRP2) (5). It is well-established that the lower limit of detection (LOD) of currently used PfHRP2-based RDTs is around 100 parasites/ $\mu\text{L}$  (6, 7). This LOD prevents identification of a significant proportion of asymptomatic individuals car-

rying lower parasitaemia levels, which can account for more than 50 % of the parasite rates observed in the population (8, 9).

Increasingly, the presence of *pfhrp2/pfhrp3* gene deletions in circulating *P. falciparum* populations that result in false negative PfHRP2-based RDTs is being reported, particularly in South America and Sub-Saharan Africa (10). On the contrary, there are also reports of false positive malaria RDTs. The high stability of the PfHRP2 antigen leads to persistence of this antigen circulating in blood up to several weeks post malaria treatment (11–13). An additional limit of RDTs is that based on the species restricted expression of PfHRP2 to *P. falciparum*, only presence of *P. falciparum* can be measured. Although there are RDTs available which use antibodies targeting Pan-*Plasmodium* antigens such as lactate dehydrogenase or aldolase, their sensitivity is lower than PfHRP2-based RDTs (5, 12). *P. malariae* and *P. ovale* infections which are often co-endemic with *P. falciparum* are usually characterized by very low parasitaemia (14) and will rarely be detected by RDTs.

Nucleic Amplification Techniques (NATs) for *P. falciparum* diagnosis, such as polymerase chain reaction (PCR), are more sensitive and more specific than RDTs (15). These techniques were adapted to detect non-*falciparum Plasmodium* species. The use of NAT-based diagnostic tests for detection and identification of malaria parasite species adds substantially more information to malaria control and surveillance programs (16). The commonly used procedure of blood sample collection for NAT based malaria diagnostic assays includes collection and storage of capillary blood on filter papers and is particularly suitable in resource-limited settings (17). Recently, a protocol co-extracting RNA and DNA (herein referred to as nucleic acids, NA) from dried blood spots (DBS) was published which allows the ultrasensitive detection of malaria parasites based on reverse transcription quantitative PCR (RT-qPCR) (18).

Over the past decade several reports have been published describing the use of DNA from RDTs for molecular characterization of malaria infections (studies reviewed in Table 1).

Apart from malaria parasites, it was also demonstrated that RDTs could serve as reliable source of NA for the detection of dengue virus (19) and antibiotic-resistant typhoid (20). The use of RDTs as a source for NA that can be used as an input for NATs would have several advantages, including the small amount of blood and cost-effectiveness regarding sample collection as well as simplified storage and shipping conditions under room temperature (RT). However, most of the studies were small, between 40 and 855 RDTs included and focused rather on demonstrating the feasibility of extracting DNA than fitting this approach for molecular surveillance of malaria parasites at larger scale.

Here, we developed systematically and evaluated extensively a procedure to extract NA from RDTs that have been used for malaria surveillance. We demonstrate that our ENAR (**E**xtraction of **N**ucleic **A**cids from **R**DTs) protocol identifies and quantifies *Plasmodium* spp. infections by amplifying the extracted NA using RT-qPCR. Our approach is supported by custom-made software solutions allowing to analyse thousands of RDTs in a standardized and high-throughput manner.

## Material and methods

### School-based survey in Mkuranga district

Carestart<sup>TM</sup> HRP2/pLDH Combo RDTs (Access Bio, Inc., Somerset, NJ, USA) were used to determine the parasite rate in three primary schools in the Mkuranga district of Coastal Tanzania. Extraction protocol A, which is based on the Quick-DNA<sup>TM</sup> Miniprep Kit (Zymo Research Corporation, Irvine CA, USA), was used to extract DNA for this school-based survey. *Plasmodium* spp. was detected by amplifying the pan-*Plasmodium* 18S rDNA (21, 22) followed by the identification of *P. falciparum* using telomere-associated repetitive element 2 (PfTARE2) (23) and the acidic terminal sequence of the var genes (PfvarATS) (23). *P. ovale* spp. was identified based on amplification of the reticulocyte binding protein 2 (PoRbp2) (24) and *P. vivax* based on the amplification of *P. vivax*-specific 18S rDNA (Pv18S) (25). Two independent assays were used to screen for the presence of *P. malariae* parasites, based on plasmepsin 4 (PmPlasp4) (26) and a novel *P. malariae*-specific multicopy target (PmRep3). For the PmRep3 assay, 2 µL extracted DNA was added to 8 µL of qPCR reaction mix. The qPCR reaction mix contained 1x HOT FIREPol® EvaGreen® qPCR Mix (Solis BioDyne, Tartus, Estonia), 0.2 µM each forward (5'-TTCATACTTAACTGTAAGC-3') and reverse (5'-GTTTCAGAAMWTTTGAGGG-3') primers. Amplification and qPCR measurements were performed using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). The thermal profile used for PmRep3 qPCR is as follows: 15 min at 95 °C; 45 cycles of 15 s at 95 °C and 60 s at 55 °C. Specificity was assessed based on the melting temperature of the amplicon, which was 71.7 °C for *P. malariae*.

### **Sample collection, analysis and storage during CHMI studies**

RDTs were collected during two CHMI studies conducted to evaluate Sanaria's PfSPZ Vaccine in Bagamoyo, Tanzania. The first CHMI was conducted in 2016 (referred to CHMI-1, ClinicalTrials.gov Identifier: NCT02613520) the second CHMI was conducted in 2018 (referred to CHMI-2, ClinicalTrials.gov Identifier: NCT03420053). Fresh venous whole blood collected in EDTA tubes was pipetted onto the RDTs within 45 min after blood collection. During CHMI-1, 20 µL was applied to BinaxNOW® Malaria RDT (Alere, Cologne, Germany) and during CHMI-2, 5 µL was applied to Carestart™ HRP2/pLDH Combo RDT (Access Bio, Inc., Somerset, NJ, USA). The RDTs were read according to the manufacturers guide and then stored in a box at RT until extraction of nucleic acids.

The same samples were used to monitor parasitemia during CHMI by thick blood smear microscopy and qPCR as described elsewhere (27, 28). All samples were processed and analysed at the laboratories of the Bagamoyo branch of the Ifakara Health Institute in Tanzania.

### **Nucleic acid extraction methods from RDTs**

The cassettes of the RDTs were opened, the entire RDT strip removed, and cut into four small pieces in order to fit into a 1.5 mL microcentrifuge tube. A set of cleaned forceps and scissors were used with special attention given to the prevention of cross-contamination between samples. After processing sample, scissors and forceps were cleaned in 10% sodium hypochlorite and wiped with ethanol sprayed tissues and dried before processing the next sample. The four nucleic extraction protocols tested, named A-D, were all based on silica columns and are described below.

### **Protocol A - Quick-DNA™ Miniprep Kit**

Protocol A is based on the Quick-DNA™ Miniprep Kit (Zymo Research Corporation, Irvine CA, USA). Briefly, 1 mL of Genomic Lysis Buffer was added to the pre-cut RDT strip in a 1.5 mL microcentrifuge tube. Samples were incubated at 95°C for 20 minutes. The supernatant was transferred onto the extraction column and the manufacturers guide was followed for extraction. DNA was eluted in 50 µL of DNA Elution Buffer. In the modified version of protocol A the RDT strips were incubated at 60°C for 60 minutes.

### **Protocol B – Quick-DNA™ Miniprep Plus Kit**

Protocol B is based on the Quick-DNA™ Miniprep Plus Kit (Zymo Research Corporation, Irvine CA, USA). Briefly, 600 µL of Solid Tissue Buffer (Blue) and 40 µL of Proteinase K was added to the pre-cut RDT strip in a 1.5 mL microcentrifuge tube and incubated at 55°C for 60 minutes. The supernatant was transferred to a clean 1.5 mL microcentrifuge tube and 640 µL of Genomic Lysis Buffer was added and mixed thoroughly. The mixture was then transferred onto the extraction column and the manufacturers guide was followed for extraction. DNA was eluted in 50 µL of DNA Elution Buffer. The modified version of protocol B had an increased incubation duration of 180 minutes.

### **Protocol C – NukEx Pure RNA/DNA Kit**

Protocol C is based on NukEx Pure RNA/DNA Kit (Gerbion GmbH, Kornwestheim, Germany), which co-extracts DNA and RNA. Briefly, a working solution of 500 µL of Binding Buffer, 4 µL of Poly A and 50 µL of Proteinase K was added to the pre-cut RDT strip in a 1.5 mL microcentrifuge tube and incubated at 60°C for 10 minutes. The supernatant was transferred onto the NukEx Spin Column and the manufacturers guide was followed for extraction. Total nucleic acids were eluted in 50 µL of Elution Buffer. The modified version of protocol C has an increased incubation duration of 180 minutes.

### **Protocol D – Zainabadi *et al.* extraction method for DBS**

Protocol D is based on a recently published extraction protocol for total nucleic acids from dried blood spots (18). Identical buffer compositions were used and the protocol was adapted to extraction of NA from RDT strips. Briefly, the pre-cut RDT strip was incubated in 900 µL lysis buffer at 60°C for 120 minutes. The supernatant was transferred onto Omega HiBind RNA Mini Columns (Omega Bio-Tek, Norcross, USA) and nucleic acids extracted as described. Total nucleic acids were eluted in 50 µL of Elution Buffer (Quick-DNA™ Miniprep Kit, Zymo Research Corporation, Irvine CA, USA).

### **High-throughput extraction protocol of nucleic acids from RDTs (ENAR protocol)**

We adapted protocol D to extract nucleic acids from used RDTs in a high-throughput manner. The main modification included a horizontal incubation of the entire uncut RDT strip by using sterile, RNase-/DNase-free 12-channel reservoirs (Axygen, Corning Inc, USA) and switching to a 96-well format for extraction. By removing the manual cutting of the RDT strip, the hands-on time during the extraction process is reduced, and the risk of cross-contamination by carryover during the extraction process is minimized. Up to eight 12-channel reservoirs, with a total of 96 samples, were processed in one batch. Lysis was conducted by adding 900  $\mu$ L lysis buffer to each RDT strip placed in the 12-channel reservoir followed by incubation at 60 °C for 2 hours with gentle shaking. All supernatants were then transferred to Omega E-Z 96 wells DNA plates (Omega Bio-Tek, Norcross, USA), washed with two wash buffers and finally eluted into a 96 well plate (DNA LoBind Plates, Eppendorf) with 50  $\mu$ L pre-warmed (60 °C) Elution Buffer (Zymo Research Corporation, Irvine CA, USA). With these adaptations to the protocol, nucleic acids from 96 RDTs are extracted in about three hours, with minimal hands-on time needed. One positive control (RDT spiked with 5  $\mu$ L blood containing 200 parasites/ $\mu$ L) and one negative control (Lysis buffer only) were included with each extraction plate to control for plate-to-plate consistency and cross-contamination.



### Detection of nucleic acids by qPCR and RT-qPCR assays

We used the PlasQ assay, a multiplex qPCR assay for *Plasmodium* spp. and *P. falciparum* screening and quantification (28), to analyse the NA extracted from RDTs. This assay consists of two independent *Plasmodium* targets. The Pan-*Plasmodium* 18S DNA and RNA (Psp18S) is targeted (21, 29), as well as the *P. falciparum*-specific acidic terminal sequence of the var genes (PfvarATS) (23). The human RNaseP gene (HsRNaseP) (21) served as an internal control to assess the quality of nucleic acid extraction and qPCR amplification. The PlasQ assay was run as a qPCR assay, targeting DNA only, with the same concentrations and conditions as previously described (28). In order to run the PlasQ as a RT-qPCR assay, targeting DNA and RNA templates, we added 1x Luna WarmStart RT Enzyme Mix (New England Biolabs, Ipswich, USA) and did start the qPCR program with a reverse transcription step at 55 °C for 15 min. All qPCR and RT-qPCR assays were run on a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). Samples were analysed in duplicates with positive (*P. falciparum* NF54 DNA), negative (malaria negative individual) and non-template (molecular biology grade H<sub>2</sub>O) controls. The mean C<sub>q</sub> of the two replicates was calculated and in case of one replicate interpreted as positive and the other replicate negative, then the assay had to be repeated and the result was considered positive if two positive replicates were obtained out of the total four replicates.

A RT-qPCR assay for *P. falciparum* female gametocytes was used targeting the PF3D7\_0630000 gametocyte- transcript (30). Briefly, 2 µL of extracted nucleic acids were added to 8 µL reaction mix consisting of 0.6 µM of primers, 0.3 µM probe and Luna® Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, USA). The qPCR program included reverse transcription for 15 min at 53 °C, followed by polymerase activation for 1 min at 95 °C and 45 cycles with 15 s at 95 °C and 45 s at 53 °C.

### ***P. falciparum* reference samples**

Different reference samples were used to test performance of the ENAR procedure. Experiments with reference were conducted using Carestart™ HRP2/pLDH Combo RDTs were used.

The WHO International Standard for *P. falciparum* DNA Nucleic Acid Amplification Techniques (NIBSC code: 04/176, herein referred to as PfIS) (31) was used for quantification of *P. falciparum* parasitaemia. Whole blood was spiked with different parasite densities, ranging from 10'000 to 0.1 parasites/μL and 5 μL applied onto RDT. Cultured *P. falciparum* strains PfNF54, PfDD2 and PfHB3 were diluted with whole blood to parasite densities corresponding to 1000 parasites/μL. Using these concentrations, serial dilutions were prepared and 5 μL were applied onto RDTs. Stage V gametocytes were obtained from culture as described previously (32). 5 μL gametocyte culture were applied onto RDTs and extracted using the ENAR protocol after a three-week storage at RT.

### **Data analysis and statistics**

All qPCR and RT-qPCR data generated with the ENAR protocol was uploaded to a custom-designed laboratory management and information system named ELIMU-MDx (manuscript in preparation). The ELIMU-MDx platform supports automated quality control, management and analysis of qPCR data. Data was analysed and plots created using R version 3.5.1 based on packages dplyr, ggpubr, gridextra and scales.

## Results

### **Blood stored on RDTs is a source of *Plasmodium* spp. DNA**

First, we conducted a literature search in Google Scholar of reports describing the use of DNA and RNA extracted from RDTs as input templates for NAT based molecular detection of malaria (Table 1). A total of 8 studies were published between 2006 and 2019. DNA stored on these RDTs were extracted with a variety of different extraction methods. The majority of studies used the Chelex method (33) for DNA extraction and two described automated extraction systems. One study extracted DNA from the entire RDT strip, all other studies used only predefined fragments of the RDT strip. In summary, these studies demonstrated that *P. falciparum* DNA can be recovered from RDTs and amplified then by nested PCR or qPCR. In these studies, most RDTs had been probed with blood from RDT or microscopy positive patients or even febrile clinical malaria cases, indicating high parasitaemia levels. Beside the identification of *P. falciparum* parasites, the DNA isolated from RDTs was used for drug resistance screening and assessing the multiplicity of infection (Table 1).

First, we employed RDTs collected in a school-based malaria survey conducted in three primary schools in the Mkuranga district of Coastal Tanzania, as study to test feasibility of using DNA on RDTs for molecular malaria surveillance. RDTs collected from 190 children from two primary schools, in Bupu and Mkerezange, with high (56.7% and 44.3%, respectively) and one primary school in Ngunguti (8.3%) with low parasite prevalence as assessed by RDT were used. DNA from RDTs was extracted following protocol A and human DNA was successfully amplified from all 190 RDTs. *P. falciparum* DNA was recovered in 91.4% (64/70) of PfHRP2-positive RDTs as detected by the PfvarATS assay. Two additional children were positive by the PfTARE2 assay. Two out of the four remaining children with PfHRP2-positive RDTs but negative for *P. falciparum* DNA self-reported to have received antimalarial

treatment during the past two weeks. Additionally, the PfvarATS assay detected *P. falciparum* DNA in 11.7% (14/120) of negative RDTs. *P. falciparum* was the most prevalent *Plasmodium* species, followed by *P. malariae*. These two species were found in children from all three primary schools, while *P. ovale* spp. was found in only two RDTs, both collected in Bupu (Figure 1A).

Based on the *P. falciparum*-specific PfvarATS qPCR assay, the parasite densities were estimated in the blood collected on RDTs (Figure 1B). RDTs which were negative did have the lowest *P. falciparum* parasitaemia levels, followed by RDTs positive for PfHRP2. RDTs positive for both antigens, PfHRP2 and pLDH, had the highest parasitaemia levels. These results reflect the well known LODs for PfHRP2 (100 Pf/μL) and pLDH (1000 Pf/μL). Notably, the lowest parasite density found was 7.6 Pf/μL, indicating that detection based on DNA extracted from RDTs has a reduced sensitivity among infections with ultra-low (< 1 parasite/μL) parasite densities.

### **Comparison of protocols for nucleic acid extraction from RDTs**

Encouraged by the outcome of the school-based survey, we aimed to improve the extraction method of NA from RDTs further. The Cq value of the human *rnasep* gene (HsRNaseP target), which is the internal control of the previously published PlasQ assay (28), was used to assess the overall performance of the NA extraction procedure. Four different extraction protocols were compared. Protocol A and B are limited to isolation of DNA (Fig 2A), while protocols C and D extract both RNA and DNA (Fig 2B). For the DNA only extraction procedures, the performance of protocol B, which uses an extraction buffer for solid tissues, was significantly better than protocol A (more than 4 Cq difference), which is our standard protocol for extracting DNA from whole blood. Protocol modification, including increased incuba-

tion times did not improve the performance of either of the protocols (not shown). Among the methods capable of co-extracting DNA and RNA, protocol D did yield more NA than protocol C. Lower C<sub>q</sub> values were observed when RNA is also targeted for amplification using RT-qPCR in samples extracted with protocol D, although the difference was not statistically significant ( $p=0.14$ , paired Wilcoxon test).

Side by side comparison of DNA extracted by using protocol A from the Tanzanian school-based survey with a selection of 71 RDTs collected in Bagamoyo and extracted with protocol D confirmed the superior performance of protocol D based on the detection of DNA only ( $p<0.0001$ , Wilcoxon-Mann-Whitney test) (Fig 2C). In summary, side by side comparison of four protocols for extracting NA from malaria RDTs showed that protocol D performed best and was therefore renamed as ENAR (Extraction of Nucleic Acids from RDTs) and used for all following experiments.

### **Detection and quantification of *P. falciparum* parasites based on ENAR protocol**

Three RDT strips from RDTs probed with 5  $\mu$ L fresh whole blood and six RDTs probed with 5  $\mu$ L frozen whole blood samples were cut into three pieces, containing the sample pad (proximal part), the detection area (middle part) or the absorption pad (distal part). Each of these three parts was used to extract nucleic acids separately, followed by analysis with PlasQ RT-qPCR assay (Fig 3A). In RDTs probed with fresh blood, *P. falciparum* NA are found in all three parts, with more than 87% of total DNA enriched in the middle part. RDTs probed with frozen blood that is associated with red blood cell lysis resulted in a more equal distribution of NA along the RDT strip.

The ability of the ENAR protocol to extract RNA is demonstrated with the following experiment. 5  $\mu$ L of an *in vitro* generated gametocyte culture was applied to RDTs and stored at RT for three weeks before NA were extracted. The gametocyte-specific transcript

PF3D7\_0630000 was reverse transcribed and amplified using a published assay which does not require DNase treatment for specific detection of gametocytes (30). Extracted NA from 5  $\mu$ L undiluted and 1:100 diluted culture amplified specifically the gametocyte marker, while the control without reverse transcription step did not result in amplification (Fig. 3B).

One of the advantages of qPCR assays are the possibility to quantify parasitaemias. Therefore, the 1st WHO International Standard for *P. falciparum* DNA Nucleic Acid Amplification Techniques (PfIS) was used to explore the feasibility of quantifying *P. falciparum* parasites. Different parasite densities, ranging from 0.1 to 10'000 parasites/ $\mu$ L of the PfIS, were spiked into whole blood and 5  $\mu$ L of this mixture was pipetted onto RDTs. A total of 56 individual RDTs, with different parasitaemia levels were extracted and analysed with the RT-qPCR PlasQ assay (Fig 3C). A reversed correlation between parasitaemia levels and Cq values were observed for both targets, the *P. falciparum* specific PfvarATS and the pan-*Plasmodium* target Pspp18S. 18S ribosomal RNA, detected by the Pspp18S assay, is highly and constant expressed during the lifecycle of *Plasmodium* spp. (34, 35), while the acidic terminal sequence of the var genes (PfEMP1), detected by the PfvarATS assay, has lower expression levels (36). The Pspp18S based assay detects total NA while the PfvarATS assay is limited to amplification of DNA only. Based on the slope, RT-qPCR efficiencies of 75.4% and 124.3% were calculated for PfvarATS and Pspp18S, respectively. RDTs negative for PlasQ assay amplification ( $C_q > 45$ , colored in red) carried mostly dilutions representing parasitaemia levels  $\leq 1$  parasite/ $\mu$ L. Two exceptions were observed where the Pspp18S assay failed to amplify two RDTs probed with higher parasitaemia levels (5 and 10 parasites/ $\mu$ L, respectively). RDTs probed with 1 parasite/ $\mu$ L were detected in 4 (PfvarATS) and 7 (Pspp18S) out of 10 RDTs. The higher detection rate at low parasitaemia levels for Pspp18S compared to PfvarATS is an indicator for the co-amplification of DNA and RNA in these samples.

We also evaluated the ENAR protocol with cultured *P. falciparum* strains including PfNFDD2, PfHB3 and PfNF54 by preparing serial dilutions in whole blood with parasite densities corresponding to 0.1 – 1000 Pf/μL (Fig 3D). RDTs were then probed with 5 μL of this mixtures and NA extracted. In all three cultures and using RT-qPCR, the PfvarATS assay did not amplify the NA from RDTs containing 0.1 parasites/μL, while the Pspp18S assay was able to amplify 0.1 Pf/ μL in two out of three *P. falciparum* strains. If analysed by qPCR, parasite densities of 0.1 parasites/μL were not detected.

In summary, NA from *P. falciparum* are highly concentrated in the middle part of the RDT when fresh whole blood is applied. RNA can be preserved and extracted from RDTs as shown with the detection of the gametocyte-specific transcript PF3D7\_0630000 and increased sensitivity of the Pspp18S RT-qPCR assay, which amplifies the highly abundant 18S rRNA. Quantification of parasitaemia can be performed, based on a correlation between parasitaemia levels and Cq values obtained from the RT-qPCR assays. The lower limit of detection (LOD) for RT-qPCR-based amplification of NA from RDTs is around 1 parasite/μL, although 10x lower parasitaemia levels can be detected as demonstrated with the freshly cultured PfNFDD2, PfHB3 and PfNF54 strains.

## **Evaluation of ENAR protocol using Controlled Human Malaria Infection studies as a platform**

Controlled Human Malaria Infection (CHMI) has been shown to be safe, well tolerated and is now regarded as a reliable tool to test protective efficacy of vaccine candidates in clinical trials (37, 38). qPCR is increasingly used in CHMI studies, also in malaria endemic countries, to closely monitor asexual blood stage parasitemia levels for safety of study participants and as study endpoint (39). Blood collected from volunteers undergoing CHMI studies represent well-characterized samples with a defined parasite strain, time and dose of infection and can therefore be used to evaluate novel malaria diagnostic approaches.

The ENAR protocol was evaluated with samples collected during CHMIs assessing the efficacy of Sanaria's PfSPZ Vaccine in clinical trials in Bagamoyo, Tanzania. RDTs were used and stored as part of two CHMIs, the first CHMI was conducted in 2016 and the second CHMI in 2018 (Table 2). As part of the standard diagnostic procedures, whole blood was collected in EDTA tubes and DNA was extracted from a total of 180  $\mu$ L whole blood. DNA-based qPCR assay was run and parasitaemia quantified (defined as WB-qPCR). Parasitaemia densities as low as 0.05 parasites/ $\mu$ L are detected with the WB-qPCR protocol. All RDTs were conducted during the CHMIs with fresh whole blood from asymptomatic subjects collected 9 to 18 days post experimental malaria infection. CHMI-1 and CHMI-2 used two different types of RDTs which required 20  $\mu$ L and 5  $\mu$ L of whole blood, respectively. RDTs collected during CHMI-1 were stored 605 days on average, while RDTs collected during CHMI-2 were stored 18 days on average before processing following the ENAR protocol. During the entire storage period, RDTs were kept at RT in a closed box and protected from light and dust. During CHMI-1, RDTs were collected later (around day 14), resulting in higher parasitemia than during CHMI-2 (around day 12.7).



NA were extracted from the RDTs using the ENAR protocol and parasites were detected and quantified by qPCR and RT-qPCR using the PlasQ assay (Table 3). First, we analysed the impact of RDT storage time on the parasite detection rates. No statistically significant differences between CHMI-1 and CHMI-2 were observed for qPCR (0.56 vs 0.40, Fisher's exact test  $p = 0.29$ ) or RT-qPCR (0.67 vs 0.42, Fisher's exact test  $p = 0.07$ ). The same trends are observed, for qPCR (0.72 vs 1.0, Fisher's exact test  $p = 0.14$ ) and RT-qPCR (0.94 vs 1.0, Fisher's exact test  $p = 0.54$ ) if only blood samples with parasitemia levels  $\geq 1$  parasite/ $\mu\text{L}$  were considered. We cannot exclude the possibility that the higher blood volume, 20  $\mu\text{L}$  instead of 5  $\mu\text{L}$ , used during CHMI-1 has compensated for degradation of NA over time.

If the data of both CHMIs is combined, the overall sensitivity was 47% for qPCR and 54% for RT-qPCR when compared to WB-qPCR (Table 3). RT-qPCR is significantly more sensitive than qPCR for the Pspp18S assay (0.27 vs 0.47, McNemar test  $p = 0.0026$ ), but not for the PfvarATS assay (0.47 vs 0.47, McNemar test  $p = 1.0$ ). Interestingly, among the long-term stored RDTs collected in 2016, the detection rate of the Pspp18S assay was also significantly higher for RT-qPCR compared to qPCR (0.52 vs 0.22, McNemar test  $p = 0.01$ ). This is a strong indicator that RNA is preserved even during long term storage. False positive detections were also observed, the specificity of RT-qPCR (3 false positive) was lower than qPCR (1 false positive).

Parasitaemia levels determined by WB-qPCR versus the parasite densities obtained with the ENAR-based RT-qPCR method showed significant positive correlation (Fig 4A). The correlation was stronger with the PfvarATS assay ( $r^2 = 0.72$ ) than with the Pspp18S assay ( $r^2 = 0.39$ ). Probably because the reference parasite density assessed by WB-qPCR was based on DNA only. Interestingly, the parasite quantities estimated from RDTs with shorter storage time (CHMI-2) are closer to the reference parasitaemia levels assessed by WB-qPCR than the estimates from RDTs collected in 2016 (CHMI-1) (Fig 4B). Detection of NA from RDTs is

highly associated with parasite density in whole blood and failure in detection is associated with ultra-low parasitemia levels (Fig 4C). Interestingly, a highly similar *in vivo* parasite growth dynamics between parasitemia determined in whole blood and extracted from RDTs is observed (Fig 3D). Once parasitemia reaches a density of 1 Pf/ $\mu$ L (dashed line) the detection probability of the ENAR approach is close to 100% (Fig 4D).

## Development of a high-throughput version of the ENAR protocol

Around the ENAR protocol we wanted to develop an analysis pipeline that allows to analyse the molecular malaria epidemiology based on RDTs collected during a malaria indicator surveys (Fig 5A). First, instead of disposing the RDTs after probing and reading, they are labelled with a barcode with the intention to connect each RDT with other survey data like questionnaires or geo-referenced data (Fig 5B). As an example, we started our project with 13'505 of unsorted, barcoded RDTs collected during the 2018 malaria indicator survey on Bioko Island. For each of these barcode-labelled RDTs additional informed consent for anonymized molecular analysis was obtained from the participants. For the sorting and selection of distinct RDTs that we wanted to analyse, we developed the *RDTselect* app (40), a browser-based application which identifies barcode-labelled RDTs based on an input list containing all barcodes of a certain selection. To enable tracking of an individual RDT throughout the ENAR extraction process the *RDTallocator* app was built (41). The barcodes are scanned with a mobile phone camera and the *RDTallocator* app allocates the associated RDT strip to the next available position in a 12-channel reservoir and subsequently 96-well plate (Fig 5C). Next, without cutting the RDTs, NA are extracted using the ENAR protocol (Fig 5D). After opening the RDT shell, the entire RDT strips are incubated in a 12-well long-format plate followed by extraction in 96-well format following the ENAR protocol. All extracted samples undergo initial screening for *Plasmodium* spp. parasites and quality control using the PlasQ RT-qPCR assay (Fig 5E).

## Discussion and conclusion

The idea of using the blood stored on RDTs for molecular analysis of malaria parasites is not novel, during the past decade several protocols and approaches have been published (reviewed in Table 1). RDTs collected during epidemiological surveys provide a convenient way to get access to blood samples for molecular analysis. The small blood volume, usually 5 to 10  $\mu\text{L}$ , applied onto RDTs is a great advantage regarding blood collection in the field because it is more acceptable by participants (42) and also more secure during collection and shipping of samples (43). The small amount of blood is at the same time also a limitation. In malaria endemic populations, a high proportion of *P. falciparum* infections identified are characterized by low parasite densities undetectable by conventional diagnostic tools. Therefore, we aimed to maximize the amount of NA recovered from RDTs. First, we used the entire RDT strip to recover as much NA as possible, contrary to previous studies that used only parts of the strip thought to contain most of the NA (43–47). Second, we expanded the pool of possible target molecules for amplification to DNA and RNA by adapting a previously described extraction method of total NA (18). We found a significantly higher detection rate of malaria parasites based on amplification of the 18S ribosomal RNA using RT-qPCR compared to qPCR, most likely based on its high abundance as RNA with an estimated 10'000 copies expressed per ring form (34). Additionally, the preservation and extraction of RNA was confirmed by the detection of the gametocyte-specific transcript PF3D7\_0630000 (30) in NA extracted from 5  $\mu\text{L}$  of *in vitro* stage V gametocyte culture applied onto RDTs and stored at RT for three weeks.

We extensively evaluated the performance of the ENAR approach for detection of malaria parasites. Lower LODs, ranging from 0.02 – 50 Pf/ $\mu\text{L}$  for malaria detection based on extracted DNA from RDTs have been reported (summarized in Table 1). Using external reference

samples, such as parasites from *P. falciparum* culture or diluted blood from infected patients, to determine lower LOD, might lead to overestimated sensitivities and is reported to be difficult to reproduce (48). LODs derived from serial dilution experiments, based on external standards, might often represent the best-case scenario and not the performance under real-life conditions. We demonstrated that the LOD of the ENAR-based protocol is 10x lower if cultured *P. falciparum* parasites are used as an external reference compared to the PfIS (31), based on lyophilized blood. The availability of RDTs collected during two CHMI studies enables us to evaluate the ENAR approach under highly controlled but real-life, ex vivo conditions. Compared to parasitaemia data obtained from fresh whole blood using qPCR, the detection rate of ENAR-based RT-qPCR was found to be overall 54%. Especially in regards to the low-density *P. falciparum* infections observed during CHMI studies, this level of sensitivity is remarkable high. Side by side comparison using identical blood sample, the overall detection rate was significantly higher than detection by microscopy (9%) or PfHRP2 antigen capture by RDT (12%) or LAMP (26%). Furthermore, parasites are detected early on during blood stage infection since we found detection rate of 30% during the first week of blood stage parasitaemia using our ENAR approach. Given all the information provided, we concluded that the lower LOD should be set at 1 Pf/ $\mu$ L. This lower LOD also applies to the sensitivity of ENAR from RDTs which had been stored for longer time periods with no special preservation.

We did not find conclusive evidence that long-term storage has a negative impact on the detection rate of *P. falciparum* NA, which is similar to what previous studies have concluded (42, 43, 45, 46, 49). Contradictory, we did observe an impact of storage on parasite quantities, longer storage periods had significantly lower parasitaemia levels than RDTs which were stored for shorter periods.

Robinson *et al.*, concluded that the skew towards higher parasitaemia values derived from DBS when compared to DNA extracted from RDTs may be indicative of lower DNA quality found on RDTs (42). However, the preservation of NA from human and parasite origin on RDTs gives enough time for shipping RDTs from the field to a central laboratory at RT in a malaria endemic country. At central reference laboratories, ENAR-based extraction could be performed followed by (RT)-qPCR analyses. Blood stored on well-documented RDTs could provide for a unique and cheap biobank that will enable retrospective molecular studies over several years following distinct malaria genotypes or trends in malaria species co-infections.

We aimed to increase significantly the RDT sample extraction and analysis throughput to transform it into an amendable tool for larger scale surveillance studies. By using the entire RDT strip without cutting into pieces by scissors followed by NA extraction in the 96-well format, the processing time was reduced significantly. With the ENAR procedure, 96 RDTs can be processed in about 3 hours including the incubation time of 2 hours. Importantly, the risk of cross-contamination by carryover of DNA or RNA between samples was reduced.

In summary, our study demonstrates the feasibility of extracting and analyzing thousands of RDTs probed with blood samples in a high-throughput manner backed by smartphone apps that allow easy tracking of each RDT sample throughout the ENAR process. The costs per sample processed by the ENAR protocol are comparable to the \$1.77 per sample which was estimated for the extraction of total NA from DBS as published by Zainabadi *et al* (18). This high-throughput analysis of extracted NA will enable us to identify and quantify *P. falciparum* in various, highly diverse endemic settings. Our ENAR method could be used for the detection of *pfhrp2* and *pfhrp3* gene deletion in *P. falciparum*, the discrimination of the different *Plasmodium* species, the detection of mutations associated to antimalarial drug resistance and even other blood-borne pathogens.

**Table 1.** Review of published protocols for the extraction of nucleic acids to detect malaria parasites.

Publication/ Year	Veron <i>et al.</i> 2006 (49)	Ishengoma <i>et al.</i> 2011 (45)	Cnops <i>et al.</i> 2011 (46)	Morris <i>et al.</i> 2013 (47)	Mze <i>et al.</i> 2015 (44)	Nabet <i>et al.</i> 2016 (43)	Boyce <i>et al.</i> 2018 (50)	Robinson <i>et al.</i> 2019 (42)
Origin of RDTs	French Guiana	Tanzania	Belgium	Tanzania	Senegal, Comoros Islands	Mali	Uganda	Kenya
Storage time and temperature	6-36 months at RT	28-65 days at RT	1 week – 16 month at RT	1-9 months at RT	not specified	14 months at RT	2 years at RT	14 months at –20 °C
Number of samples for clinical evaluation	40	165	121	855	204	134	299	141
Brand of RDT used for clinical evaluation	Optimal®, ICT Now®	ParaHIT®f, Paracheck Pf®	12 different RDT brands were assessed, OptiMAL and SDFK60 were used to determine sensitivity	Paracheck-Pf, SD-Bioline Malaria Ag P.f/Pan	SD Bioline 05FK50, Malaria pLDH/HRP2 Combo	SD BIOLINE Malaria Ag P.f® and P.f/Pan®	Standard Diagnostics 05FK60 Malaria Ag P.f/Pan	SD BIOLINE 05FK60
Part of RDT used for clinical evaluation	proximal part	proximal part	proximal part	Distal part	proximal part	entire strip	distal part	central part
Extraction method	Phenol/chloroform extraction performed better than Chelex method or QIAamp DNA mini blood kit	Chelex method	Simple boil and spin method outperformed QIAamp DNA mini kit	Chelex method outperformed simple boil and spin method and ABI-based extraction	QIAamp DNA Mini kit	Automated extraction using NucliSENS EasyMAG instrument	Chelex method	Chelex method and robotic extractions using QIAAsymphony gave comparable results
Parasite detection	18S rDNA nested PCR	18S rDNA nested PCR	18S rDNA qPCR	18S rDNA nested PCR, cytochrome b nested PCR, and 18S rDNA qPCR	Nested PCR and HRM-qPCR for genotyping	18S rDNA qPCR	pfdh qPCR	pgmet-tRNA qPCR
Quantification of parasites	no	no	no	no	no	yes	no	yes
Analytical sensitivity	50 Pf/μL with 15 μL diluted blood from malaria patient	1 Pf/μL with 10 μL diluted blood from malaria patient	0.02 Pf/μL with diluted blood from malaria patient	2 Pf/μL with 5 μL diluted <i>P. falciparum</i> culture	not specified	not specified	not specified	0.05 Pf/μL based on detection on a single RDT
Clinical evaluation (detection rates)	94.3% detection rate for samples with parasite densities ≥ 400 parasites/μL	91.0% detection rate for samples positive by microscopy	100% detection rate of clinical samples with parasite densities ≥ 1 parasites/μL	No difference in detection rates compared to DBS samples from febrile patients	98.5% amplification rate for genotyping among positive RDTs	79.8% detection rate among positive RDTs collected from symptomatic febrile patients	96.0% amplification rate among RDTs from patients with severe malaria	81.25% agreement with DBS from children with positive RDT s
Non- <i>P. falciparum</i> species detected by NAT	<i>P. vivax</i>	no	<i>P. vivax</i> , <i>P. ovale</i> , <i>P. malariae</i>	no	no	no	no	no
Other application of extracted DNA	no	Antimalarial drug resistance screening ( <i>pfdhfr</i> )	no	Antimalarial drug resistance screening ( <i>pfmdr1</i> , <i>pfert</i> )	Antimalarial drug resistance screening ( <i>pfdhfr</i> , <i>pfdhps</i> ) multiplicity of infection ( <i>msp1</i> , <i>msp2</i> )	Multiplicity of infection (8 microsatellite markers)	NGS-based amplicon sequencing of <i>pfama1</i> for multiplicity of infection analysis	no

**Table 2.** Overview of blood samples collected during two CHMIs and stored on RDTs.

Study (Year)	CHMI-1 (2016)	CHMI-2 (2018)
RDT brand	BinaxNOW®	CareStart™
Sampling days post CHMI (mean and range)	Malaria RDT	Malaria (Pf/PAN) Combo
blood volume on RDT	14.0 (10.5-18.0)	12.7 (9.0-18.0)
storage time in days (mean and range)	20 µL	5 µL
storage conditions	605 (596-616)	18 (10-48)
number of RDTs collected	RT	RT
% positive by WB-qPCR	71	50
WB-qPCR parasite density (parasites/µL, geom. mean and range)	38.0% (27/71)	62.0% (31/50)
	4.7 (0.05-840.0)	0.3 (0.01-1041.0)

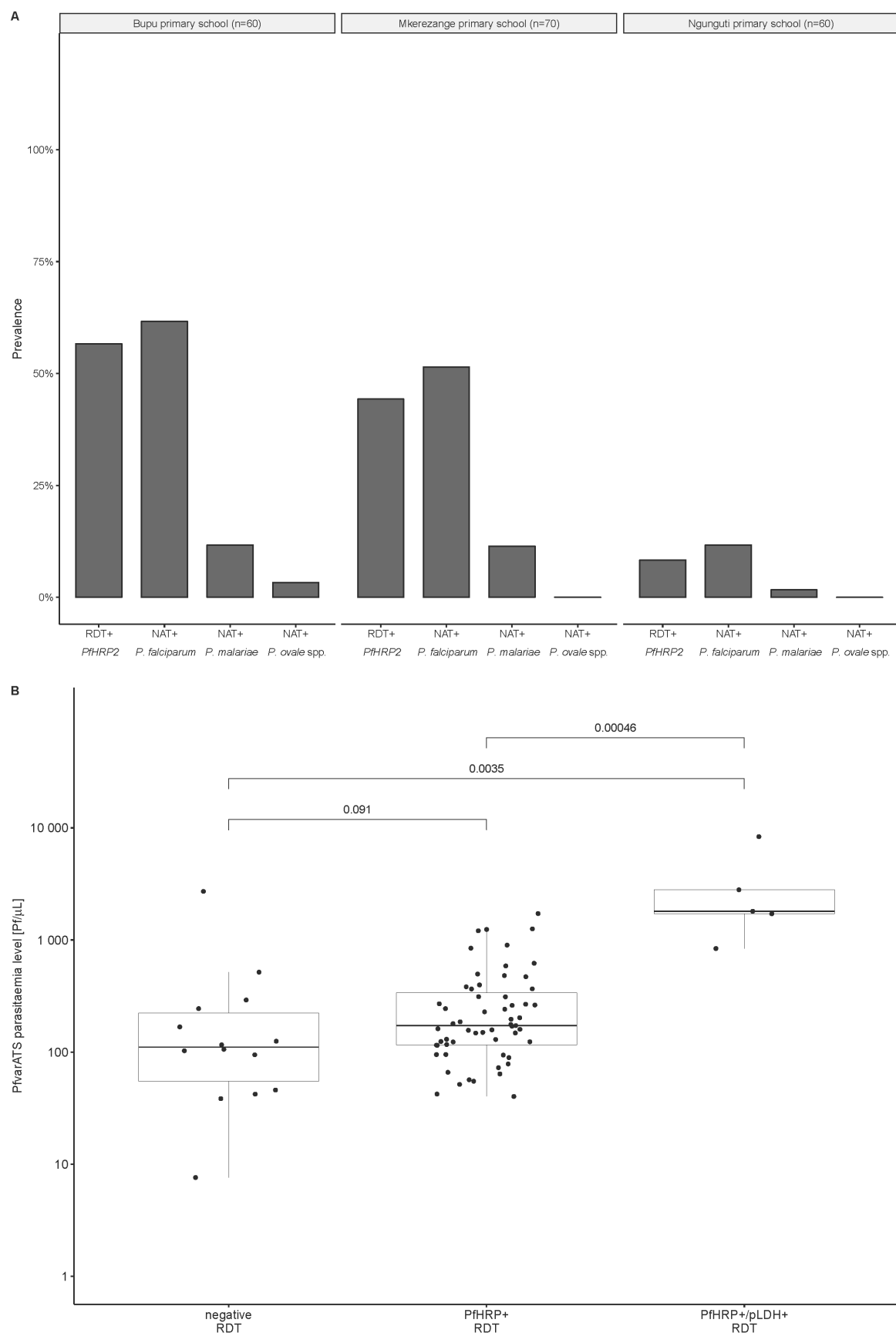
**Table 3.** *P. falciparum* detection from RDTs collected during CHMIs using the ENAR extraction protocol.

	CHMI-1 (2016)		CHMI-2 (2018)		combined	
	qPCR	RT-qPCR	qPCR	RT-qPCR	qPCR	RT-qPCR
PfvarATS detection rate <sup>a</sup>	0.56 (0.35, 0.75)	0.67 (0.46, 0.83)	0.39 (0.22, 0.59)	0.29 (0.13, 0.49)	0.47 (0.34, 0.61)	0.47 (0.34, 0.61)
Psp18S detection rate	0.22 (0.09, 0.42)	0.52 (0.32, 0.71)	0.31 (0.15, 0.51)	0.42 (0.25, 0.61)	0.27 (0.16, 0.40)	0.47 (0.34, 0.61)
PlasQ detection rate combined	0.56 (0.35, 0.75)	0.67 (0.46, 0.83)	0.40 (0.23, 0.59)	0.42 (0.25, 0.61)	0.47 (0.34, 0.61)	0.54 (0.40, 0.67)
PfvarATS detection rate (> 1 Pf/µL in WB-qPCR <sup>b</sup> )	0.72 (0.47, 0.90)	0.89 (0.65, 0.99)	1.00 (0.63, 1.00)	1.00 (0.59, 1.00)	0.81 (0.61, 0.93)	0.92 (0.74, 0.99)
Psp18S detection rate (> 1 Pf/µL in in WB-qPCR)	0.33 (0.13, 0.59)	0.78 (0.52, 0.94)	0.89 (0.52, 1.00)	1.00 (0.66, 1.00)	0.52 (0.32, 0.71)	0.85 (0.66, 0.96)
PlasQ detection rate combined (> 1 parasite/µL in in WB-qPCR)	0.72 (0.47, 0.90)	0.89 (0.65, 0.99)	1.00 (0.63, 1.00)	1.00 (0.66, 1.00)	0.81 (0.61, 0.93)	0.93 (0.76, 0.99)

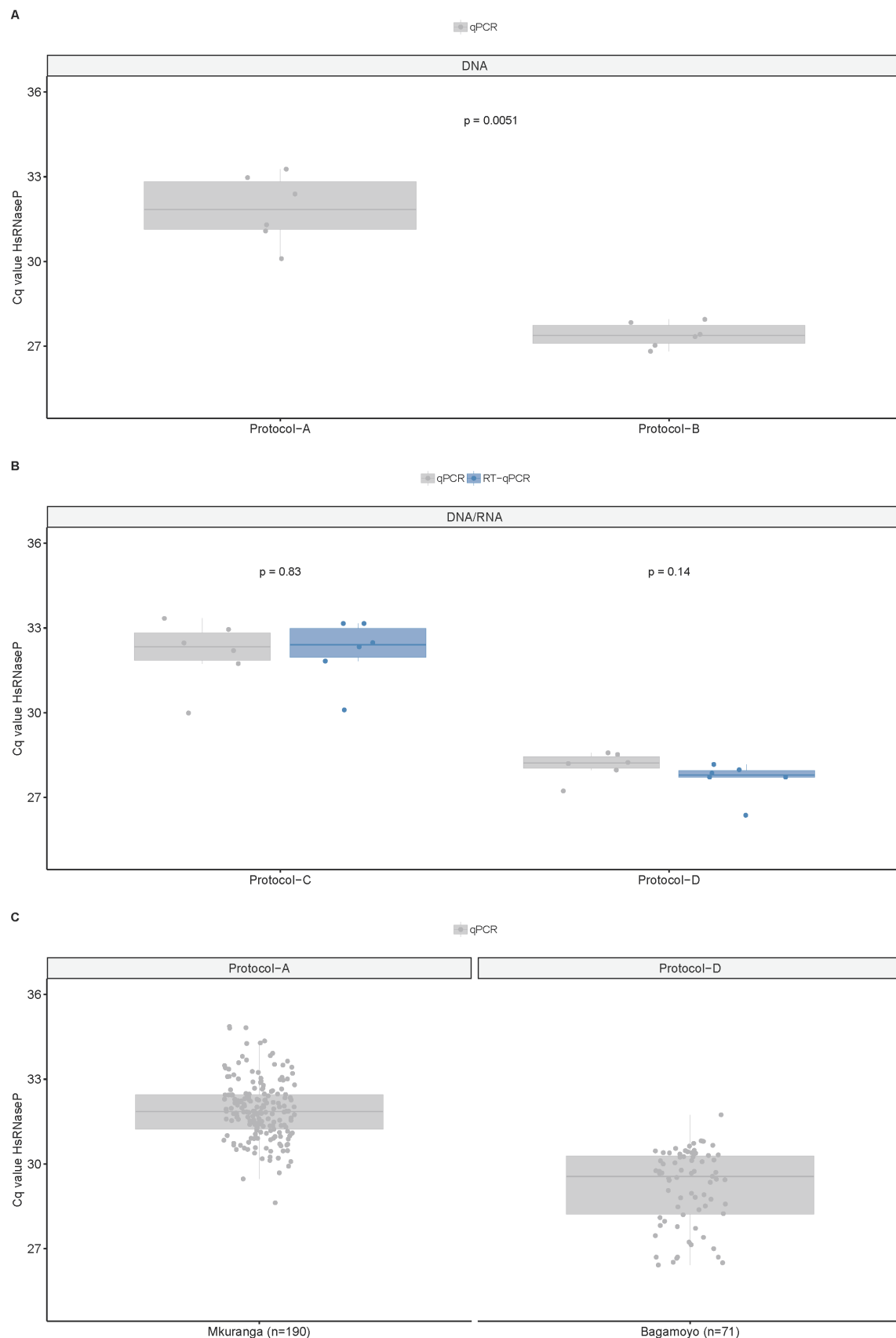
<sup>a</sup> Detection rate: proportion of detected positive samples (value between 0 and 1)

<sup>b</sup> WB-qPCR: reference parasitaemia based on qPCR detection of parasites in 180 µL of whole blood.

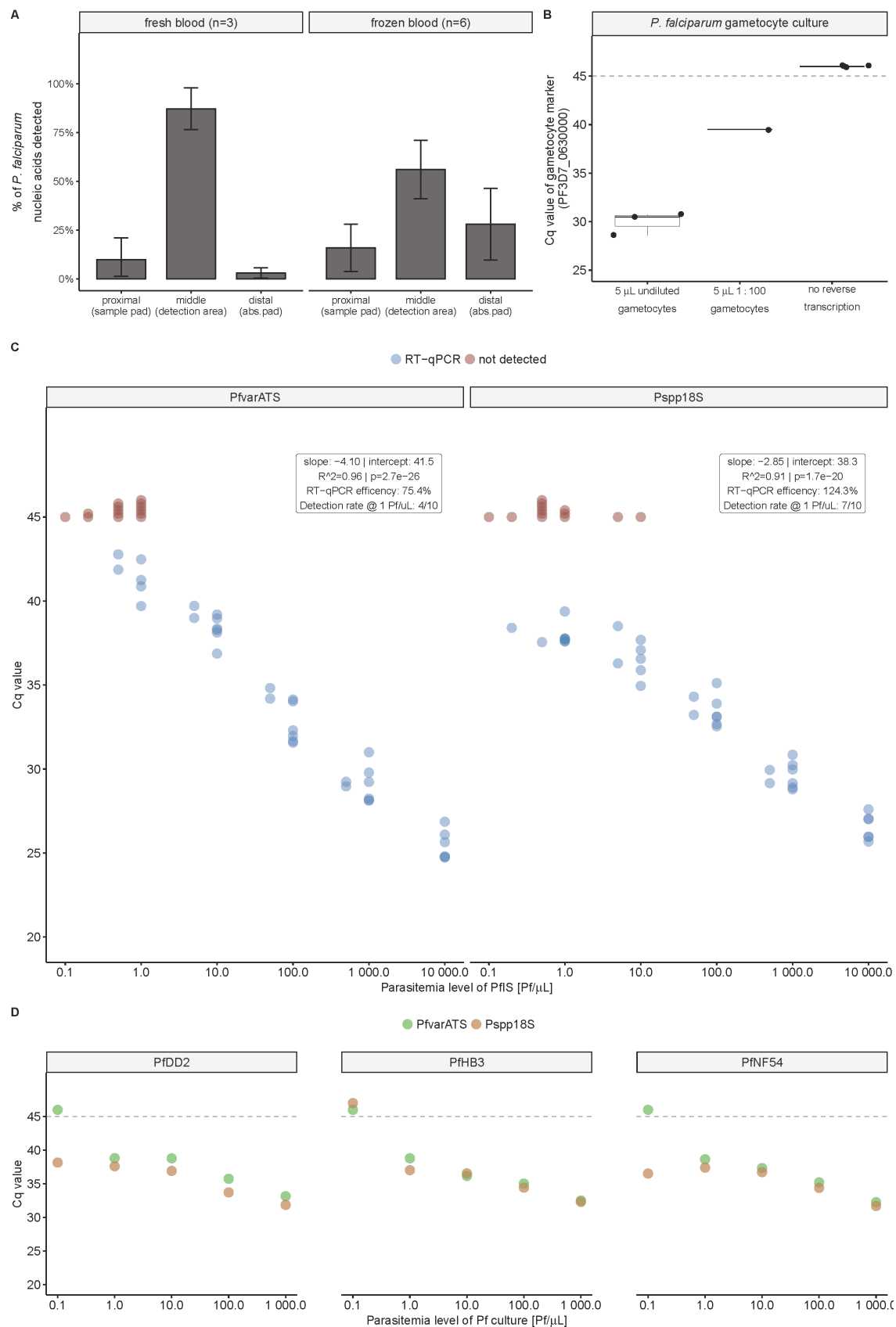




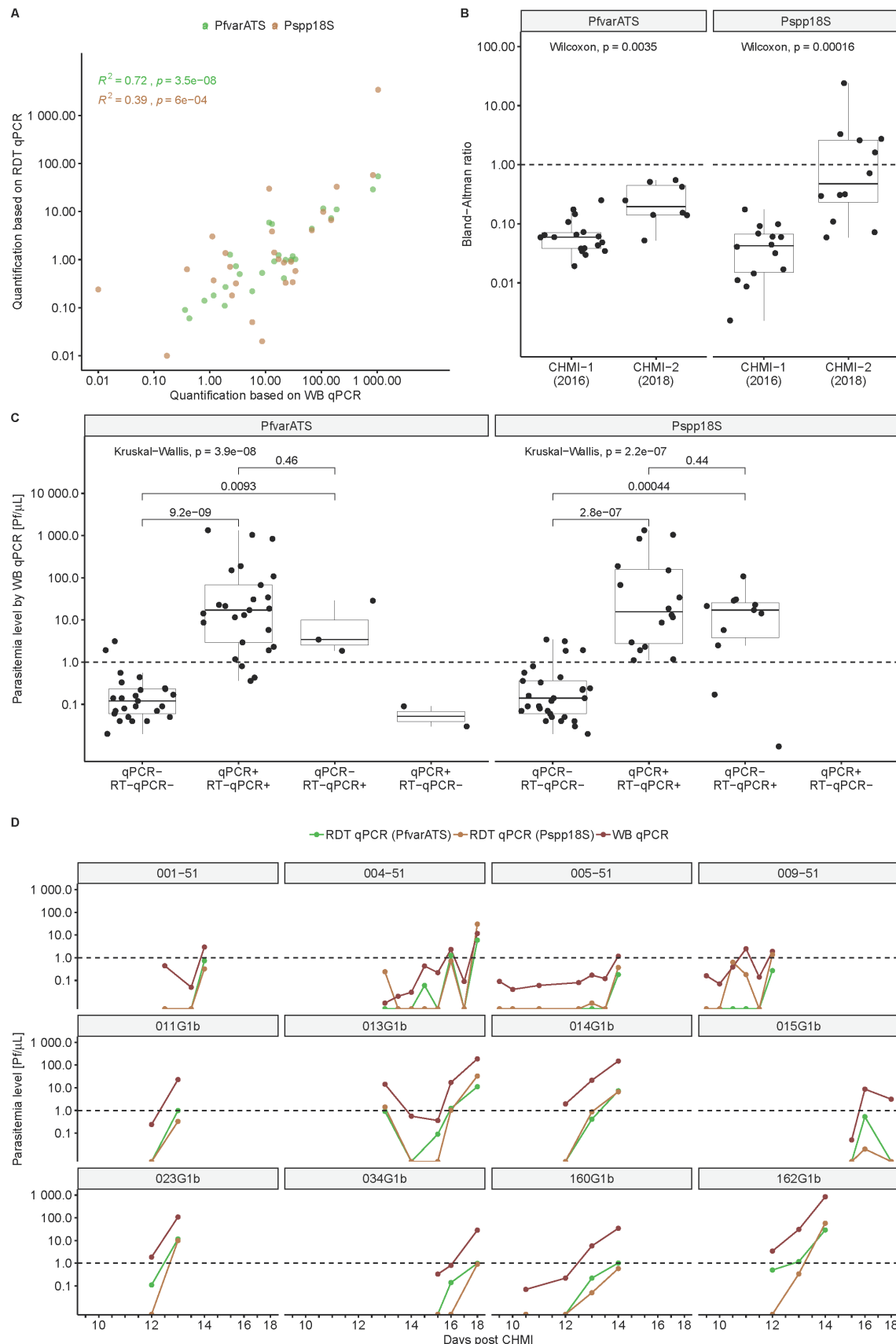
**Figure 1. Pilot study to test feasibility of extraction and detection of *Plasmodium* spp. from RDTs.** (A) Detection rates of *P. falciparum* by RDT compared to NAT-based detection of *P. falciparum*, *P. malariae* and *P. ovale* spp. from extracted RDTs collected in three primary schools in Tanzania. (B) Quantification of *P. falciparum* based on PfvarATS qPCR grouped by RDT outcome. Parasitaemia levels between groups were compared using Wilcoxon-Mann-Whitney test.



**Figure 2. Evaluation of nucleic acid extraction protocols.** (A) Comparison of protocol A and B, both extracting DNA, using the Cq value of the human *rnasep* gene as a proxy for the amount of nucleic acids extracted. Wilcoxon-Mann-Whitney test was used. (B) Comparison protocol C and D, extracting total NA, using qPCR and RT-qPCR. Wilcoxon matched-pairs signed rank test was used. (C) Side by side comparison of extraction protocols A and D in field samples collected in Mkuranga (n=190) and Bagamoyo (n=71). Wilcoxon-Mann-Whitney test was used to compare Cq values between protocol A and D.



**Figure 3. ENAR-based extraction and quantification of nucleic acids from RDTs.** (A) Identification of location of *P. falciparum* NA captured on RDTs. (B) Detection of the gametocyte-specific transcript PF3D7\_0630000 in blood on RDTs after three weeks of storage at RT. (C) RDTs containing 5  $\mu$ L whole blood spiked with various PfIS densities, ranging from 0.1 to 10,000 parasites/ $\mu$ L, were extracted with ENAR and amplified by RT-qPCR PlasQ assay. Samples which did not result in amplification are indicated in red. Assay performance characteristics are given in the box. (D) Serial dilution of cultures of laboratory-adapted *P. falciparum* strains PfDD2, PfHB3 and PfNF54 were used to determine the lower LOD for RT-qPCR detection NA extracted with ENAR.

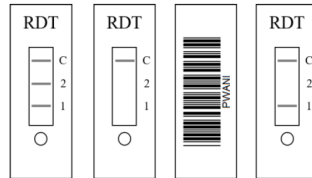


**Figure 4. Evaluating the performance ENAR approach using CHMI as a platform.** (A) Parasitaemia derived from ENAR protocol correlated to *P. falciparum* quantities determined in 180  $\mu$ l of whole blood. Spearman's rank correlation coefficient is indicated. (B) Bland-Altman ratios (ENAR/WB-qPCR) compared between the two different CHMIs (and storage periods). (C) Parasitaemia in whole blood grouped by qPCR/RT-qPCR outcomes. Groups were compared using Wilcoxon-Mann-Whitney test. (D) *P. falciparum* growth kinetic during CHMI observed in whole blood and on parasitaemia derived by ENAR approach.

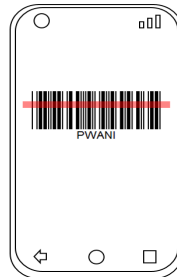
**A) Malaria indicator survey with questionnaire**



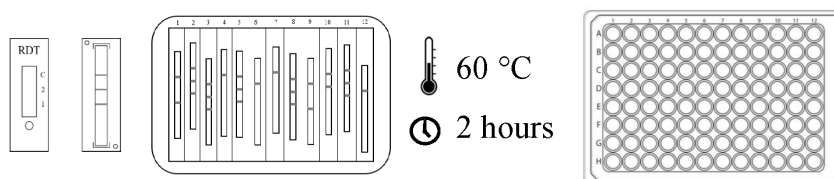
**B) Malaria diagnosis using RDT followed by storage of barcode-labelled RDTs**



**C) Smartphone-based *RDTselect* app is used to identify RDTs of interest and each RDT is allocated to a unique position in a 96-well plate using *RDTallocator* app**



**D) RDT shell is opened and nitrocellulose strip removed. Incubation of the entire strip in 12-well long-format plates followed by extraction in 96-well format using *ENAR* protocol.**



**E) Systematic *Plasmodium spp* screening and quality control of all extracted RDTs using PlasQ RT-qPCR assay.**



**Figure 5. Adaptation of ENAR protocol for analyzing large numbers of barcoded RDTs.** (A) Malaria indicator survey is conducted which includes a detailed questionnaire. (B) Malaria prevalence is determined by RDT followed by storage of barcode labelled RDTs. (C) Sorting and tracking of RDTs using smartphone apps. (D) High throughput protocol for extraction of NA from RDTs using the ENAR approach. (E) Detection, quantification and genotyping of *P. falciparum* and non-*falciparum* malaria parasite.

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## **Competing interests**

SL Hoffman is salaried and full-time employee of Sanaria Inc, the developer and sponsor of Sanaria® PfSPZ Vaccine. He was not responsible for the collection, recording or entry of the parasitological data used in this study. The other authors have no conflicts of interest.

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## **Authors' contributions**

Conceptualization: Etienne Guirou, Tobias Schindler, Claudia Daubenberger

Data curation: Tobias Schindler, Oliver Tresor Donfack

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Writing – original draft: Etienne Guirou, Tobias Schindler, Claudia Daubenberger

Writing – review & editing: Etienne Guirou, Tobias Schindler, Claudia Daubenberger

## **Ethics approval and consent to participate**

For the school-based survey in Mkranga district sample collection was approved by the Institutional Review Board of the Muhimbili University of Health and Allied Sciences and the National Institute for Medical Research, Tanzania.

The CHMI samples derived from clinical trials evaluating Sanaria's PfSPZ Vaccine. The trials were approved by the Institutional Review Board of the Ifakara Health Institute, Dar es Salaam, Tanzania, the National Institute for Medical Research, Tanzania and the Ethics Commission of Basel Land and Basel Stadt, Switzerland. Written informed consent was granted from the participants for publication of clinical and laboratory data obtained during CHMI on condition of anonymity.

Malaria Indicator Survey samples were collected in 2018 on Bioko Island, Equatorial Guinea. The survey was approved by the Ministry of Health and Social Welfare of Equatorial Guinea and the ethics committee of the London School of Hygiene and Tropical. Written informed consent was obtained from each participating adult and on behalf of participating children. Samples for which an additional consent for molecular analysis was obtained were included in this study.

## **Supporting information**

S1. Step-by-step protocol for ENAR



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# **ELIMU-MDx: A Web-Based, Open-Source Platform for Storage, Management and Analysis of Diagnostic qPCR Data**

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## **Abstract**

The **E**lectronic **L**aboratory **I**nformation and **M**anagement **U**til for **M**olecular **D**iagnostics (ELIMU-MDx) is a user-friendly platform designed and built to accelerate turnaround time of diagnostic qPCR assays. ELIMU-MDx is compliant with the MIQE guidelines and has extensive data import capabilities for all major qPCR instruments by using the RDML data standard. Designed as a web-based software tool and can run natively all major operating systems.

## **Keywords**

qPCR, RDML, MIQE, ELIMU-MDx, Diagnostic

## **Abbreviations**

qPCR (Quantitative Polymerase Chain Reaction), RDML (Real-time PCR Data Markup Language), MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments), ELIMU-MDx (Electronic Laboratory Information and Management Utensil for Molecular Diagnostics), ADF (Assay Definition File), CHMI (Controlled Human Malaria Infection), NAT (Nucleic acid Amplification Techniques)

## Background

The global market of *in vitro* diagnostics (IVD), comprising of tools to detect, diagnose, or monitor diseases, is estimated to be worth US\$ 40–45 billion (1). One of the fastest growing areas is the identification of infectious diseases using molecular diagnostics which is becoming an integral part of medical practice and public health worldwide (2). Molecular diagnostic tools, especially nucleic acid amplification techniques (NATs), provide faster, more sensitive, and often more cost-effective diagnosis than traditional culture methods for microbiological analysis (3). Quantitative polymerase chain reaction (qPCR) is a well-established method for the detection, quantification, and typing of bacteria, viruses and protozoa in the areas of clinical and veterinary diagnostics as well as food safety (4). Among the biggest advantages of qPCR-based diagnosis are the universality in designing and developing new assays as well as the widespread availability of the technology. Moreover, qPCR also allows to quantify and genotype pathogens. With the rapid spread of qPCR into routine diagnostics, standardization and validation processes of qPCR assays and data had to be addressed (4). A first step was the publication of the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (5), which has led to a certain degree of standardization in the validation process of qPCR assays across different laboratories. In order to standardize the qPCR data format the Real-time PCR Data Markup Language (RDML, [www.rdml.org](http://www.rdml.org)) was designed (6). RDML stores the raw data acquired by the qPCR instrument as well as the information required for its interpretation, such as sample annotation and cycling protocol. A comprehensive review of 27 open-access software packages and tools for qPCR data analysis reported that most tools use their own file format and only a fraction of the currently existing tools support the standardized data exchange format RDML (7). The lack of a common standard for qPCR data makes it difficult to share and analyse data across different qPCR platforms.

The high-throughput character of qPCR analysis in infectious disease diagnosis easily leads to a rapid accumulation of raw data which needs to be quality controlled and interpreted in order to correctly identify and quantify parasites. Therefore, access to well-designed, user-friendly and semi-automated software facilitating the storage, quality controlling and analysis of diagnostic qPCR data is needed.

Controlled Human Malaria Infection (CHMI), the deliberate exposure of human volunteers to infectious malaria parasites, has proven to be safe and well tolerated and accelerates the evaluation of malaria vaccines candidates in clinical trials (8, 9). Volunteers participating in CHMIs are closely monitored. Blood is drawn daily for up to two weeks and screened for *P. falciparum* parasites by thick blood smear microscopy and after the first appearance of blood-stage parasites antimalarial treatment is initiated. Nowadays, more sensitive qPCR is increasingly used in CHMI to closely monitor parasitaemia levels which improves the safety of participants (10). In order to manage the vast amount of qPCR data generated during a series of CHMI studies with more than 250 individuals, conducted in Tanzania and Equatorial Guinea, we decided to design and build a platform which facilitates the analysis and interpretation of diagnostic qPCR data. We identified several key features which in our opinion would be required for such software. I) Highly adaptable and integrative: a solution which can be easily adapted to a variety of qPCR assays, supports semi-automated data analysis and integrates into existing sample workflow. II) Data traceability throughout analysis process: the use of an audit trail and community data standards to ensure high data integrity. III) Accessibility and reliability of software: Platform-independent, open-source, and low maintenance software which could be deployed rapidly to new laboratories and other projects using qPCR analysis as monitoring tool. To address these needs, we developed the web-based, open-source platform ELIMU-MDx (Electronic Laboratory Information and Management Utensil for Molecular Diagnostics).

## Material and Methods

### ELIMU-MDx development

ELIMU-MDx combines a relational SQL database with a user-friendly web interface programmed in PHP 7.2 using the framework Laravel 5.8 and the tool Laravel Nova 2.0. The platform is accessed through the web-browser (Google Chrome, Firefox and Safari were successfully tested). It validates user provided data and stores it in a MySQL 5.7 database where appropriate constraints ensure consistency.

### Analysis of qPCR data by ELIMU-MDx

qPCR data generated for diagnostic purposes has different requirements to software than data generated for other qPCR applications. Most important, the consistency and reliability of the data needs to be ensured. Each qPCR run needs to have controls in place to test for contamination (preventing false positive results) and to test the performance of extraction and qPCR reaction (preventing false negative results). All data uploaded to ELIMU-MDx is quality controlled based on several controls and predefined criteria. Only samples which pass all QC thresholds are considered for further analysis. Additionally, a sample measured multiple times with the same assay is automatically considered as technical replicates, even if the data is derived from different experiments. This allows to repeat samples if needed.

ELIMU-MDx processes qPCR data derived from both major qPCR detection technologies. Assays using dye-based (e.g. SYBR- or Eva-Green dyes) or TaqMan-based detection can be analysed. The C<sub>q</sub> values, the number of cycles needed to reach a fluorescence threshold, is extracted from the RDML file and used for qualitative and quantitative interpretation of the qPCR assay. In case of a dye-based detection, only C<sub>q</sub> values from samples whose amplicon has a melting temperature within a pre-defined range are considered for further analysis. Since

the melting temperatures are not included in the RDML version 1.1 from Bio-Rad CFX Maestro (Version 4.1.2433.1219) and RDML version 1.2 from MyGo Pro PCR Software (Version 3.4), they need to be uploaded separately in addition to the RDML file.

The current version of ELIMU-MDx supports absolute quantification of the qPCR target molecules using the slope and intercept derived from the linear relationship between C<sub>q</sub> values and a calibration curve generated by using serially diluted standards of known concentrations.

The Assay Definition File (ADF) includes all assay-specific parameters which together define the assay. The advantage of defining all assay parameters in one single file is its universality and easy adaption to new qPCR assays. An example for an ADF is provided as excel file (supplementary file 1).

## Results

### ELIMU-MDx overview

The structure of ELIMU-MDx is shown in Figure 1. Basically, ELIMU-MDx consists of three modules, the sample module, the assay module and the data module which are connected by an experiment. Each of the modules is supplied with data and parameters from specific components. These components contain sample, assay or data specific information which are necessary to interpret and document the experiments. The ADF, which contains all parameters necessary to analyse samples measured with the appropriate qPCR assay, is linked through the assay to the experiment. The experiment is considered the central node of ELIMU-MDx and combines the sample and assay metadata with data. Upload of standardized data is followed by initial quality control and automated analysis and eventually results in a downloadable result file which can be used for generating reports or statistical computing and data visualization by any statistical software.

Examples of the ELIMU-MDx user interface are illustrated in Figure 2. All features are accessible through a sidebar (Fig 2A). The oligo details are shown as an example for the level of detail which is provided by ELIMU-MDx virtual freezer (Fig 2B). As part of the built-in sample inventory, the storage position of each sample can be displayed (Fig 2C). Results are automatically displayed after uploading the qPCR RDML files and samples with inconclusive results can be easily identified using the custom-build filters (Fig 2D). The integrated audit trail records all major events and therefore ensure high data integrity (Fig 2E).

### **ELIMU-MDx is built as an open-source web-app**

ELIMU-MDx is a user-friendly platform designed and built to accelerate turnaround time of diagnostic qPCR assays. The advantages of web applications include rapid deployment, cross-platform compatibility and easy application development (11). A detailed guide to setup the platform, with step-by-step instructions, is provided at <https://sparclex.github.io/elimu>. The low-maintenance structure is an additional advantage, particularly in resource-limited settings. The source code is open source and distributed under a MIT license and is publicly available for download. It is unlike most commercially available qPCR software not a black box. All features of ELIMU-MDx are completely disclosed and can be improved or changed by other member of the scientific community or even used separately in other software.

The ELIMU-MDx can be accessed through <https://elimu.ch> where we have created a specific user account for demonstration purposes (login: `demo.user@elimu.ch`, password: `elimu2019`).

### **ELIMU-MDx uses the RDML data standard making it qPCR instrument independent**

ELIMU-MDx supports RDML, a structured and universal data standard for exchanging qPCR data. RDML files contain all information to understand the experimental setup, re-analyse the data and interpret the results. The use of the RDML data standard allows to use data from different qPCR instruments. The platform was successfully tested with RDML files version 1.1 and 1.2 from the Bio-Rad CFX96 Real-Time System and the MyGo Pro qPCR instrument.



### **The semi-automated data quality control and analysis improves sample-to-result turnaround times**

The use of the standardized data format of RDML files allowed us to automate quality control, analysis and validation of qPCR data. Initial quality control includes the automated analysis of the Positive Control (PC), Negative Control (NC) and Non-template Control (NTC) of each qPCR run uploaded based on provided cut-off values. The internal control (IC) of each sample is used to validate the extraction and amplification performance. Based on the C<sub>q</sub> values provided by the RDML file and a set of assay specific parameters, as defined by the ADF, quality control as well as qualitative and/or quantitative analysis is performed.

The ELIMU-MDx platform was extensively tested with diagnostic qPCR data generated during CHMI studies in Tanzania (total of 478 samples) and Equatorial Guinea (total of 2907 samples). We observed a significant reduction in time spent on the quality control review process of the qPCR data when compared to our previously used custom-built Microsoft Access database.

### **ELIMU-MDX simplifies documentation, statistical analysis and visualization of qPCR experiments**

A qPCR experiment, as defined by the ELIMU-MDx, contains all information required by the MIQE guidelines. The electronic laboratory notebook-like feature of ELIMU-MDx documents and links information from the sample module, the assay module and the data module for each experiment and sample. Additionally, assay-specific information is provided by the ADF (Table 1).

The ELIMU-MDx platform also serves as a laboratory information and management system (LIMS). We added general features of a LIMS to the ELIMU-MDx platform. A unique storage position can be assigned to each sample in order to keep track of sample storage. If samples are shipped, all details of the shipment will be logged. Apart from qPCR data, all other types of results can be uploaded and attached to the samples and therefore linked with the qPCR result. Importantly, the structured data generated by ELIMU-MDx allows to use the R language and environment for statistical computing and visualization of qPCR data without extensive data manipulation and cleaning.

### **Integrity and traceability of qPCR data in ELIMU-MDx**

All qPCR data uploaded to ELIMU-MDx is protected from accidental or intentional modifications. The qPCR raw data, as a RDML file, is attached to the results. The Cq values cannot be changed or deleted, the only option is to exclude replicates. Exclusion of Cq values is visually indicated and can be reversed. Each modification of the data is attributed to a user. The use of personal accounts which are password protected and the automatic creation of an audit trail which records and logs all major changes are in line with the FDA regulations on electronic records and electronic signatures (FDA regulation 21 CFR Part 11).

## Discussion

The qPCR represents a powerful tool which has spread in different areas of infectious disease diagnostics. The technology has become indispensable for the detection of viruses, bacteria or protozoa and increasingly replaces traditional diagnostic techniques (4). For laboratories, especially if they generate diagnostic data for clinical trials the adequate storage, curation and analysis of qPCR data can be a challenge. Currently available software solutions, commercial or open-source, are not well suited for the management and analysis of diagnostic qPCR data. Often a combination of several tools is used with an inadequate documentation of this essential step of the qPCR workflow.

We have built the ELIMU-MDx platform as a response to the large amount of qPCR data generated during clinical trials including CHMI studies. Due to its integration into the sample workflow and automation of the qPCR QC and preliminary analysis the use of ELIMU-MDx accelerates the sample-to-result turnaround time significantly. The use of the RDML standard allows to analyse the qPCR data in a streamlined and transparent way without losing the association between the final result (interpretation of the qPCR data) and the actual raw data (fluorescent data of the qPCR run). We also placed importance on the documentation of the qPCR experiments by attaching metadata from the samples and assays to the qPCR data.

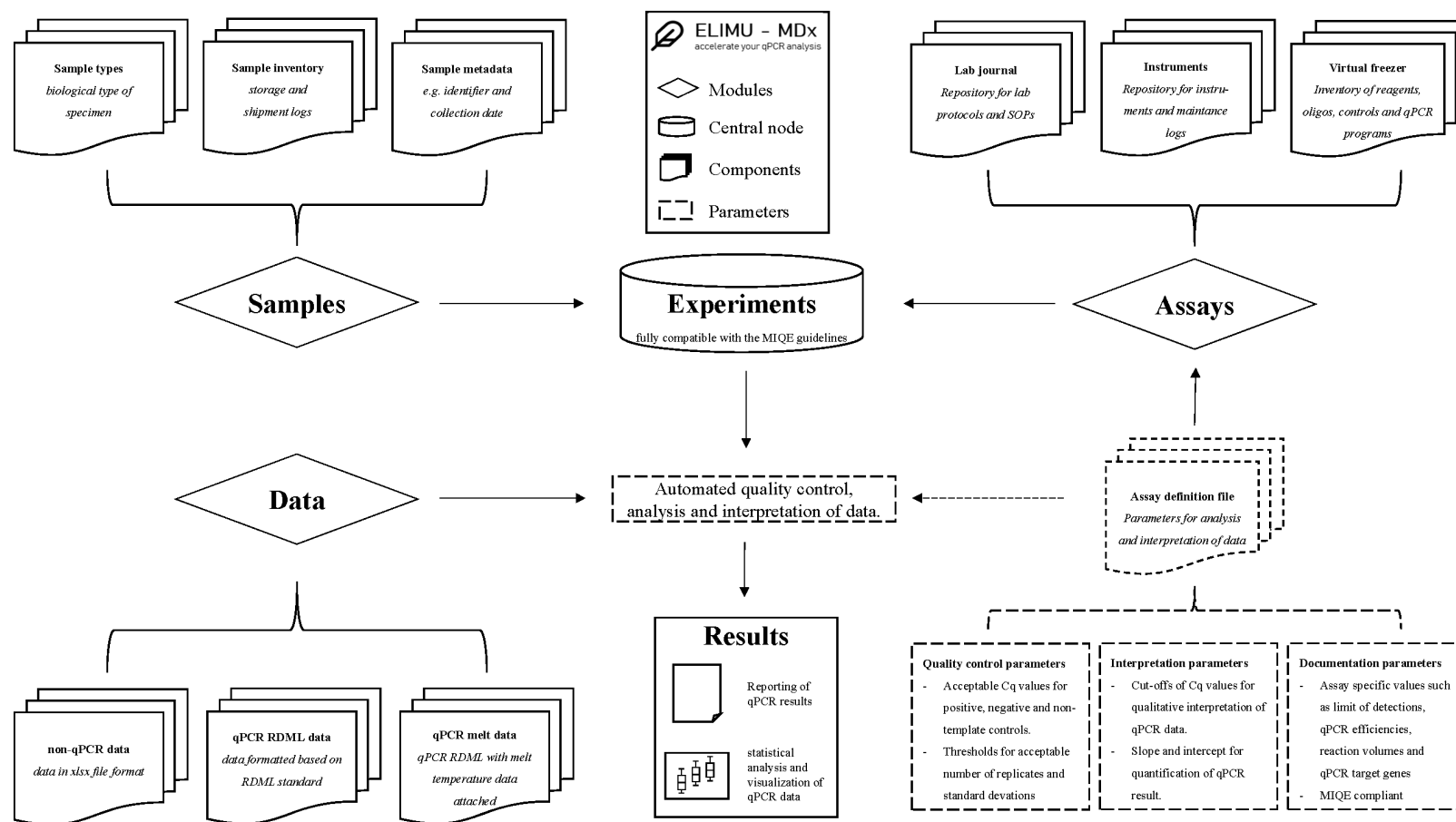
ELIMU-MDx is open source licensed under the permissive MIT license. Disclosing the software code used for data analysis should be the standard and will definitely help to increase the reproducibility of data analysis (12). Because ELIMU-MDx is an open-source project, contributions and feedback from the community is highly encouraged and will be an important factor in improving ELIMU-MDx.

## Conclusion

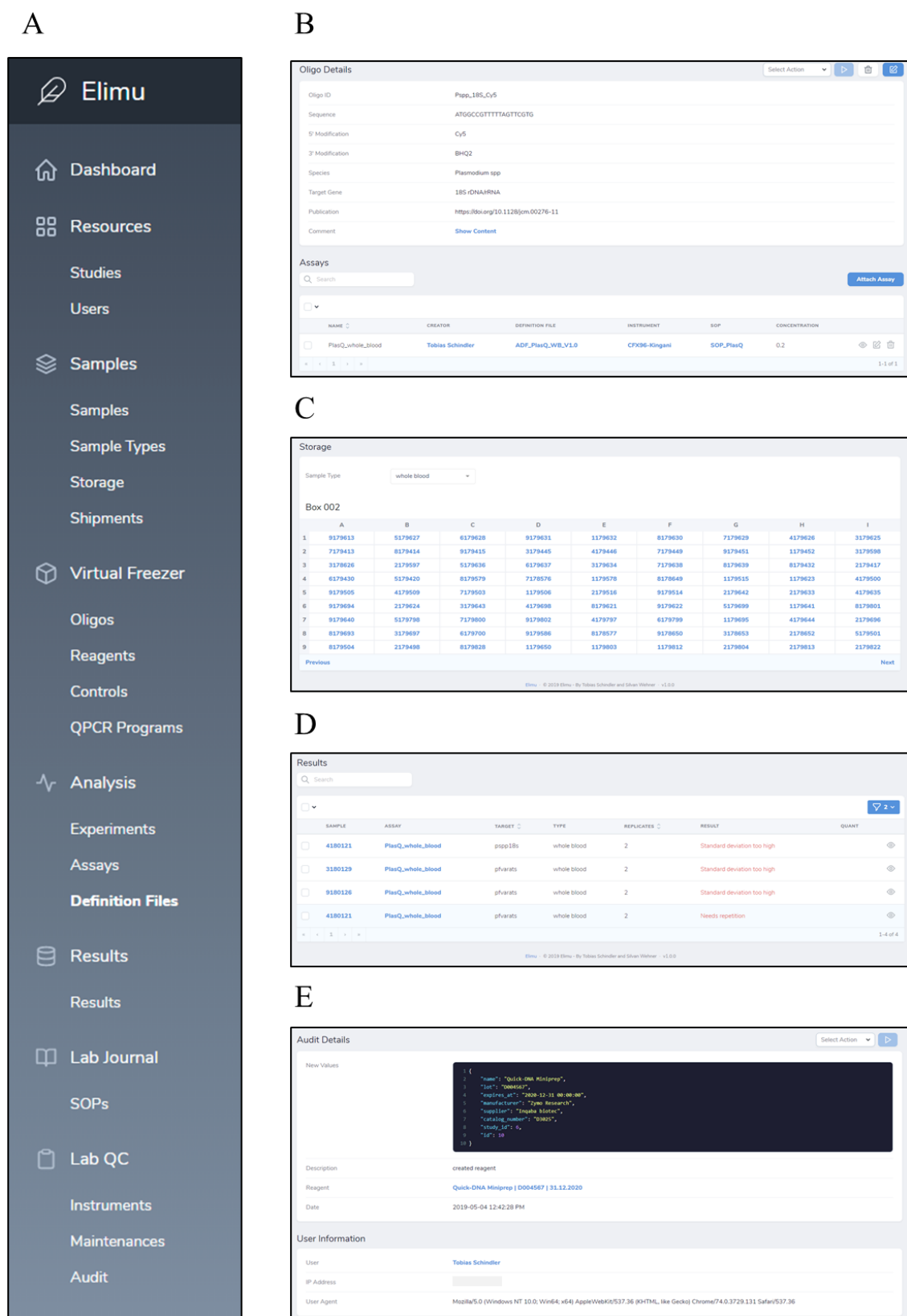
ELIMU-MDx has the potential to connect all qPCR-data associated processes in a laboratory environment. Starting from documenting the qPCR experiment using its electronic laboratory notebook-like feature, to large-scale qPCR data management and collection. Finally, researchers can analyse the pre-processed and cleaned qPCR data using the third-party software solutions of their choice and perform final statistical analysis. All these steps are combined in a user-friendly and open-source web-application which is compliant with the MIQE guidelines.

**Table 1.** Information documented for each experiment and sample.

Modules	Component	Description
Sample module	Sample metadata	Required is a unique identifier, collection date and biological type of sample. Optional information such as demographics can be added.
	Sample inventory	Contains information related to storage and shipment of samples.
	Lab journal	Contains detailed information for the associated protocols and standard operating procedures (SOPs).
	Instruments	A detailed repository for qPCR instruments, which also includes maintenance logs.
Assay module		Contains information for:
	Virtual freezer	<ul style="list-style-type: none"> <li>- Oligonucleotides: sequences, link to original publication.</li> <li>- Reagents: qPCR reagents with lot number and expiry date and extraction kits for nucleic acids.</li> <li>- Controls: name and concentration of positive Controls.</li> <li>- qPCR programs: Cycling conditions and detection channels used. qPCR instrument software name and version.</li> </ul>
Data module	qPCR raw data	Cq values and melting temperatures provided by RDML file.
	qPCR metadata	Metadata for each run are saved within the RDML file.
Assay Definition File	Analysis parameters	Assay-specific parameters which are used to analyse and interpret the qPCR data. For details refer to the ELIMU-MDx user guide.
	Documentation parameters	Additional assay-specific parameters, e.g. qPCR efficiencies, which are used for documentation purposes only.



**Figure 1. Structure of ELIMU-MDx.** The sample, assay and data modules are connected to create an experiment, which is the central node of the platform. Assay-specific parameters are provided through the Assay Definition File (ADF).



**Figure 2.** User interface of ELIMU-MDx. (A) Side bar for navigation within ELIMU-MDx. (B) Oligo details as an example for an ELIMU-MDx component. (C) Automatically assigned storage position for samples. (D) Samples which need special attention can be filtered in the results section. (E) ELIMU-MDx audit trail.

## **Availability and Requirements**

Software name: ELIMU-MDx

Software home page: <https://www.elimu.ch>

Software source: <https://github.com/Sparclex/elimu>

Open-source license: MIT license

Operating systems(s): OS independent, designed as a web-app Supported browsers: Firefox, Google Chrome and Safari.

Programming languages: PHP

Other requirements: <https://sparclex.github.io/elimu/dev-docs/requirements.html>

Any restrictions to use by non-academics: no limitations

## **Supporting Information**

S1. Example of assay definition file (*xlsx* file)



### **Authors' contributions**

Concept and design: SW, FS, CD, TS. Programming: SW. Beta testing, troubleshooting and providing feedback: PM, EG, AD, SH, MM, SM, TS. Drafting the manuscript: SW, TS, CD. All authors read and approved the final manuscript.

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## Chapter IV

Active surveillance of *P. malariae* and *P. ovale* spp. infections in Tanzania and Equatorial Guinea

Chapter 4 includes data on active surveillance of asymptomatic *P. malariae* and *P. ovale* spp. infections in Tanzania and Equatorial Guinea. In collaboration with the central blood bank in Malabo, Equatorial Guinea we analysed 200 blood donations for the presence of *Plasmodium* spp. parasites by qPCR. In order to identify the *Plasmodium* species infecting the blood donors, we developed a novel multiplex qPCR assay for parallel malaria parasite identification. Using this assay, the main five human infectious malaria species can be detected qualitatively in a single tube rendering it highly cost efficient. We found that more than one quarter of the blood donations contained malaria parasites and that 75% of *P. falciparum* and 100% of *P. malariae* and *P. ovale* spp. infections were missed by RDT and microscopy.

We also published a report, describing two cases of *P. malariae* infections that were identified during a study evaluating the safety, tolerability and efficacy of the PfSPZ Vaccine in Bagamoyo, Tanzania. Since these two participants were enrolled into a clinical trial, we were provided with a unique opportunity to study clinical manifestations of *P. malariae* over four months of follow up period.

This chapter contains the following publications:

Schindler *et al.* **Molecular monitoring of the diversity of human pathogenic malaria species in blood donations on Bioko Island, Equatorial Guinea.** *Malaria Journal* 2019


Schindler *et al.* **Two cases of long-lasting, sub-microscopic *Plasmodium malariae* infections in adults from coastal Tanzania.** *Malaria Journal* 2019

RESEARCH

Open Access



# Molecular monitoring of the diversity of human pathogenic malaria species in blood donations on Bioko Island, Equatorial Guinea

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## Abstract

**Background:** Malaria can be transmitted by blood transfusion from human to human and it is responsible for the majority of transfusion-transmitted infectious diseases worldwide. In sub-Saharan Africa, it had been estimated that almost a quarter of blood donations contain malaria parasites. Since rapid diagnostic tests and thick blood smear microscopy lack sensitivity for low density parasitaemia, particularly in asymptomatic adults, the most reliable method to assess the problem of transfusion-transmitted malaria are nucleic acid-based molecular approaches such as quantitative polymerase chain reaction. The study was undertaken to determine the prevalence of sub-microscopic malaria parasite infection among blood donors in Malabo, Equatorial Guinea.

**Methods:** Between July and August 2017, a total of 200 individual blood samples from blood donors at the Malabo Blood Bank were collected and screened by rapid diagnostic tests and thick blood smear microscopy. Retrospectively, the same samples were analysed for the presence of undetected, low-density malaria parasites using quantitative polymerase chain reaction.

**Results:** In comparison to 6.5% (13/200) by rapid diagnostic test and 2.0% (4/200) by microscopy, the proportion of *Plasmodium falciparum* positive blood donations analysed by quantitative polymerase chain reaction was significantly higher (26%, 52/200). Densities of *P. falciparum* positive blood donations were ranging from 0.06 to 3707.0 parasites/ $\mu$ L with 79.6% below 100 parasites/ $\mu$ L and therefore not detectable by non-molecular malaria diagnostic tests. qPCR based species identification revealed that *P. falciparum* was the dominating species responsible for 88.1% (52/59) of positive blood donations, followed by *Plasmodium malariae* (15.3%, 9/59) and *Plasmodium ovale* (3.4%, 2/59).

**Conclusions:** This study confirms that in malaria endemic settings, sub-patent malaria infections among blood donors are prevalent. In blood collected from healthy donors living in Malabo, *P. falciparum*, *P. malariae* and *P. ovale* parasites were identified. Currently widely used malaria diagnostic tools have missed more than 75% of *P. falciparum* containing blood donations, demonstrating the value of quantitative polymerase chain reaction to reliably detect low density *P. falciparum* infections. Since the availability of molecular diagnostic methods in malaria endemic countries is still limited, the blood recipients living in malaria endemic countries should be treated following WHO recommendations.

**Keywords:** Transfusion-transmitted malaria, *P. falciparum*, *P. malariae*, *P. ovale*, qPCR

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## Background

Malaria is a vector-borne parasitic tropical disease found in 55 countries globally [1]. Six *Plasmodium* species are known to infect humans including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri* and *Plasmodium knowlesi* [2]. Malaria is transmitted to the human host through the bite of a female *Anopheles* mosquito when the sporozoite stage is inoculated during the feeding. The parasite undergoes a pre-erythrocytic liver stage reproduction that is clinically silent and lasts 1 to 2 weeks before the release of merozoites into the bloodstream. These merozoites rapidly infect red blood cells, starting the asexual blood stage whereby the parasite undergoes cycles of asexual replication that include the ring, trophozoite, and schizont stages leading to regular fever episodes [2]. In adult human populations living in malaria endemic countries, the prevalence of sub-patent malaria infections is up to 80% [3] with certain parasite clones surviving in the human host more than 300 days [4].

Diagnosis of malaria is performed in blood samples collected from potentially infected humans. The gold standard for malaria diagnosis remains light microscopy of stained blood smears. Thick blood smears provide sensitivity and thin smears allow the identification of different malaria species and the quantitation of infection [5]. Rapid diagnostic tests (RDTs) are widely used since they offer less dependence on the availability of laboratory infrastructure, and can also be employed by inexperienced health workers [6]. RDTs are often based on parasite-derived histidine-rich protein 2 (PfHRP2) for sensitive and specific detection of *P. falciparum*, and lactate dehydrogenase enzyme (LDH) or aldolase for a *Pan-Plasmodium* detection of all human infective malaria species [7]. The lower limit of detection (LOD) for microscopy is between 50 and 500 parasites/ $\mu$ L depending on the expertise of the microscopist [5] and 100 parasite/ $\mu$ L for the PfHRP2 based *P. falciparum* RDTs [8]. Differential diagnosis of *P. ovale*, *P. malariae*, *P. vivax* and *P. knowlesi* is hampered by the low density blood stage infections and the difficulty in distinguishing these species based on morphology of trophozoites using microscopy [9].

Nucleic acid amplification technologies (NATs) are more expensive and require advanced laboratory infrastructure but provide much better sensitivity. The parasites' multi-copy 18S small subunit ribosomal deoxyribonucleic acid (rDNA) and/or its highly expressed ribonucleic acid (rRNA) is the most frequently used biomarker for NATs [10]. Different methods for 18S rDNA/rRNA detection are currently in use with a great variation in sensitivity. Qualitative methods targeting 18S

rDNA, such as nested polymerase chain reaction (PCR) [11] and LAMP [12], reach LODs of 0.1–10 parasites/ $\mu$ L. The use of quantitative PCR (qPCR) [13], does not only allow absolute quantification of infections but also lowers the LOD to less than 1 parasite/ $\mu$ L. The use of Reverse Transcription quantitative PCR (RT-qPCR), amplifying total nucleic acids (RNA and DNA) of the 18S genes [14–16], and qPCR assays targeting alternative multi-copy genomic sequences [17] further increase sensitivities of NATs. Apart from increased sensitivity of NATs, molecular detection using PCR has improved species discrimination compared to either microscopy or RDTs [18].

Screening for transfusion transmitted infections should follow the World Health Organization (WHO) recommendations which include searching for chronic infectious diseases including HIV-1, HIV-2, Hepatitis B, Hepatitis C and *Treponema pallidum* (syphilis) [19]. Based on the local epidemiological infectious disease situation, the WHO recommends that blood donations should also be screened for malaria [19, 20]. Malaria can be efficiently transmitted by blood transfusion from human to human and is undoubtedly responsible for the majority of transfusion transmitted diseases in the world [21]. The rate of transfusion-transmitted malaria (TTM) in malaria endemic sub-Saharan regions is estimated between 14 and 28% [22]. Several reports describe that *P. falciparum* [22], *P. vivax* [23], *P. malariae* [24], *P. knowlesi* [25] and *P. ovale* [26] can be transmitted either through blood donations or solid organ transplantations.

Malaria species circulating in Equatorial Guinea include *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* [27–29]. The Bioko Island Malaria Control Project (BIMCP) started in 2004 with the aim to reduce malaria transmission and to control the burden of disease and has received substantial funding from the Government of Equatorial Guinea and private donors including Marathon EG Production Limited, Noble Energy, and Atlantic Methanol Production Company. The malaria control strategy of the BIMCP rests on vigorous vector control measures, effective case management, prevention of malaria during pregnancy, behavioural change communications and regular monitoring and evaluation [30]. The BIMCP has had significant success in reducing *P. falciparum* parasite prevalence in the 2–14 years old children and adolescent from 45% (95% CI 40–50%) in 2004 (baseline) to 12.1% (95% CI 11.2–13.3%) in 2016 based on annual Malaria Indicator Survey (MIS) data [30].

Since RDTs and microscopy lack sensitivity for low-level parasitaemia, particularly in asymptomatic adults, the most reliable method to assess the problem of TTM are NATs such as qPCR. This study has set out to analyse a selection of 200 blood samples from the blood bank in Malabo using qPCR to identify sub-patent *P. falciparum*

infections that cannot be detected by commonly used RDTs and microscopy. In addition, the presence of other TTM causing *Plasmodium* species was analysed using a newly developed single tube multiplex qPCR assay.

## Methods

### Study site and participant recruitment

The study was conducted at the Central Blood Bank in Malabo on Bioko Island. Bioko Island, located in West-central Africa and home of Equatorial Guinea's capital city Malabo, is 32 km from the coast of Cameroon. The approximately 250,000 people, who mainly reside in Malabo are at risk of malaria year-round [30]. Adults willing to donate blood and who are Hepatitis B, Hepatitis C, and HIV negative were eligible for blood donation. Microscopy and RDT (CareStart™ Malaria HRP2/pLDH Pf/PAN Combo) were used for malaria screening at the blood bank and the results were kept at the blood bank until the end of qPCR analysis. The Malabo Central Blood Bank processes around 100 donors per month; the majority being donors assisting friends and families during emergency situations, and the remainder are donors that voluntarily donate blood on a regular basis. The blood bank is run by a public–private cooperation between the Ministry of Health and Social Welfare, the University of Valencia Hospital, and funded by the AGEM-GUINEA company.

### Sample collection for molecular diagnostics

Between July and August 2017, a total of 200 individual blood donations from routine visitors were selected to conduct this study. 1 mL of whole blood was collected in EDTA tubes as part of the usual blood donation procedures in the blood bank. The blood was immediately used for microscopy and RDT, with the remaining blood immediately stored at  $-20^{\circ}\text{C}$ . The samples were transported in a cooling box to the laboratory of the Equatorial Guinea Malaria Vaccine Initiative (EGMVI) in Malabo. The laboratory infrastructure of the EGMVI, located on the premises of the La Paz Hospital in Malabo, which conducted the first clinical trial in the history of the country [31], was used for qPCR analysis of the blood donations.

The 200 blood samples, from healthy, Hepatitis B, Hepatitis C, and HIV-1 and HIV-2 negative blood donors were analysed for the presence of possibly undetected, low-level malaria parasites using high-sensitive qPCR assays. DNA extraction was done manually from 180  $\mu\text{L}$  whole blood using Quick-DNA Miniprep kits (Zymo Research, Irvine, USA) following manufactures' guidelines and DNA was eluted with 50  $\mu\text{L}$  Elution Buffer. DNA samples were kept at  $-20^{\circ}\text{C}$  prior to qPCR analysis.

### qPCR for *Plasmodium* screening and quantification (PlasQ assay)

*Plasmodium* spp. and *P. falciparum* parasites were quantified using the PlasQ assay. This assay consists of two independent *Plasmodium* targets combined in a multiplex assay. The *Pan-Plasmodium* 18S rDNA sequence (Psp18S) [13, 32], and the *P. falciparum*-specific acidic terminal sequence of the *var* genes (PfvarATS) [17] were used for detection and quantification of parasites. The human RNaseP (HsRNaseP) gene served as an internal control to assess the quality of DNA extraction and qPCR amplification.

Amplification and qPCR measurements were performed using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). The thermal profile used for PlasQ qPCR is as follows: 60 s at  $95^{\circ}\text{C}$ ; 45 cycles of 15 s at  $95^{\circ}\text{C}$  and 45 s at  $57^{\circ}\text{C}$ . Each reaction contained 2  $\mu\text{L}$  DNA and 8  $\mu\text{L}$  reaction master mix containing 1 $\times$  Luna Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, USA) and 1 $\times$  PlasQ Primer Mix (Additional file 1). All qPCR assays were run in duplicates with appropriate controls including Non-Template Control [NTC] and *P. falciparum* 3D7 DNA as positive control [PC]. The mean Cq of the two replicates was reported and in case of one qPCR replicate interpreted as malaria positive and the other replicate negative, then the assay had to be repeated to arrive at an unequivocal result.

Using the first WHO International Standard for *P. falciparum* DNA Nucleic Acid Amplification Techniques (NIBSC code: 04/176), a serial dilution ranging from 0.05 to 10,000 parasites/ $\mu\text{L}$  was prepared and used for quantification of *P. falciparum*. The WHO standards were run as duplicates in two out of the total 13 qPCR runs conducted.

### qPCR for *Plasmodium* species identification (PlasID assay)

Several published [17, 33–37] and unpublished qPCR assays, detecting *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax* and *P. knowlesi* were evaluated and the best performing primer and probes for each *Plasmodium* species were combined into a new multiplex assay (PlasID). The primers and probe combinations used for this novel pentaplex malaria qPCR assay is provided in Additional file 1.

Amplification and qPCR measurements were performed using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). The thermal profile used for the PlasID qPCR is as follows: 15 min at  $95^{\circ}\text{C}$ ; 45 cycles of 15 s at  $95^{\circ}\text{C}$  and 60 s at  $55^{\circ}\text{C}$ . Each reaction contained 2  $\mu\text{L}$  DNA and 8  $\mu\text{L}$  reaction master mix containing 1 $\times$  HOT FIREPol® Probe qPCR Mix Plus



(Solis Bio Dyne, Tartu, Estonia) and 1× PlasID Primer Mix (Additional file 1). All qPCR assays were run in duplicates with appropriate controls including Non-Template Control [NTC] and DNA of six *Plasmodium* species as positive controls [PC]. DNA controls for *P. falciparum*, *P. knowlesi*, *P. malariae*, *P. vivax*, *P. ovale curtisi* and *P. ovale wallikeri* were used to evaluate species specificity of the PlasID assay. The positive controls were reconfirmed by two commercial qPCR assay (GeneFinder™ Malaria RealAmp Pf/Pv/Pk and Pf/Pm/Po). Samples with a Cq value less than 45 for any of the five *Plasmodium* targets were considered positive for the corresponding *Plasmodium* species.

### Data management and analysis

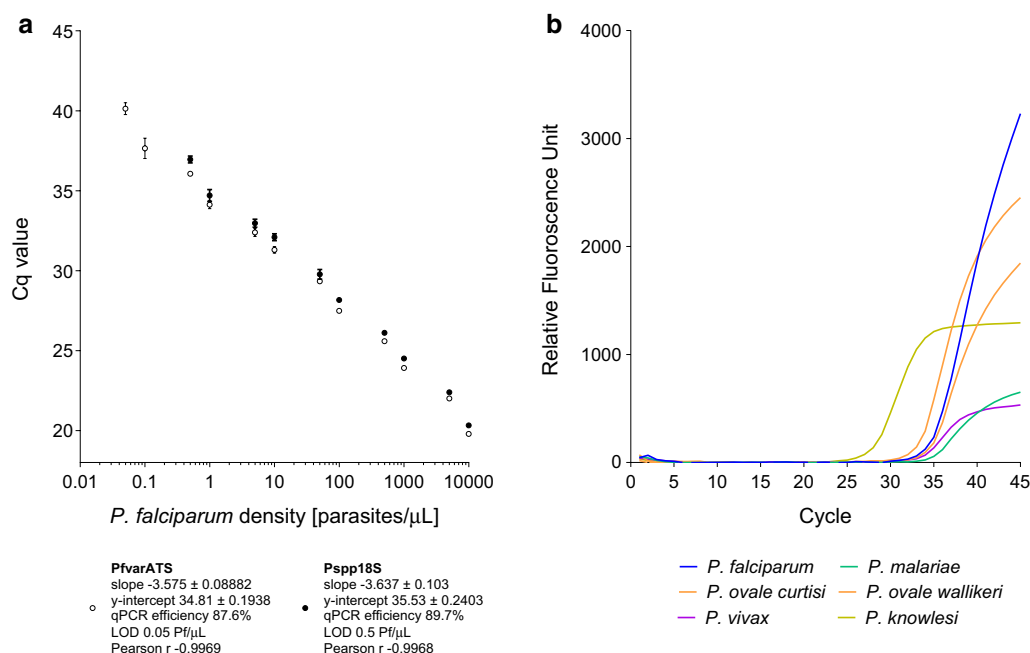
Geneious version 8.1.9 (Biomatters Ltd, Auckland, New Zealand) was used for sequence alignments and oligonucleotide designs. Cq values were obtained from the Bio-Rad CFX96 Manager 3.1 software (Bio-Rad Laboratories, California, USA) and transferred to a Microsoft Access based in-house database designed for storage and analysis of qPCR data. Cq values were transformed to parasite densities and linked to patient's metadata collected at the blood bank. Categorical variables were compared by Fisher's exact test and continuous variables by Mann–Whitney using GraphPad Prism version 7.00 for

Windows (GraphPad Software, La Jolla, USA). P values < 0.05 were considered to be significant.

## Results

### Development and implementation of multiplex qPCR assays for malaria screening

Two independent qPCR assays were systematically used for the screening and identification of malaria parasites. The use of two consecutive qPCR assays maximizes the amount of information generated by the analysis. The first assay (PlasQ), applied to all samples, was designed to identify and quantify *P. falciparum* and/or non-*falciparum* species. Additionally, the internal control serves as a general control for quality and performance of the DNA extraction and qPCR reaction. The performance of the PlasQ assay is shown in Fig. 1a. For both targets, PfvarATS and Psp18S, the Cq values for different parasite densities, ranging from 0.05 to 10,000 parasites/μL were obtained (Pearson  $r = -0.9969$  and  $-0.9968$ , respectively). Similar qPCR efficiencies for both targets were observed (87.6% and 89.7%). The PfvarATS target did detect two additional samples carrying low parasite densities (0.1 and 0.05 parasites/μL) resulting in 10 times lower LOD compared to the Psp18S target. The second assay (PlasID), which is applied to all *Plasmodium* spp. positive samples, was designed for rapid identification



**Fig. 1** Analytical performance of PlasQ (**a**) and PlasID (**b**) assay. **a** Correlation of *P. falciparum* standards and the Cq values for both targets, Psp18S (black circles) and PfvarATS (white circles) of the PlasQ assay. Based on these quadruplicate replicates of the WHO standards, LODs and qPCR efficiencies were calculated. **b** The ability of the PlasID assay identifying five different malaria species is shown by a representative, composite amplification plot. DNA from the six *Plasmodium* species were analysed in separate tubes during the same qPCR experiment

of five different malaria causing *Plasmodium* species. Performance of the PlasID assay was assessed using well-defined clinical samples as references and this novel assay's ability of identifying *P. falciparum*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. vivax* and *P. knowlesi* is demonstrated in Fig. 1b.

#### Baseline characteristics of blood donors attended Malabo Central Blood Bank

Blood donor characteristics are given in Table 1. All donors were Equato-Guineans living in Malabo on Bioko Island except two persons, one originating from neighbouring Douala, Cameroon and the other from the United States. The majority were male (88.5%) with a median age of 32 years, ranging from 18 to 57 years. Seventy-seven donations were from voluntary blood donors and 123 donations were from replacement donors, who donate blood when required by a member of the patient's family or community (Table 1). Except for the blood pressure, no significant difference in relation to gender, age, weight, pulse and anaemia status between these two donor groups were observed.

#### Prevalence of malaria positive blood donations

The flow diagram depicting the sequential malaria diagnostic tools applied to the blood donations is summarized in Fig. 2. In the Malabo blood bank, all samples were screened using thick blood smear microscopy and RDT. Microscopy identified four *P. falciparum* positive donations, while RDTs detected 13 malaria positive donations. Three donations were positive for both antigens, the *P. falciparum* specific HRP2 and the *Plasmodium* spp. LDH antigens. The remaining ten donations were positive for HRP2 only. Microscopy and RDT positive blood donations were not considered for donation

and were included blinded into this study sample collection on purpose to test the performance of the PlasQ assay.

Upon arrival at the EGMVI laboratory all samples were screened with the first multiplex qPCR assay (PlasQ), targeting *P. falciparum* (PfvarATS), *Plasmodium* spp. (Psp18S) and the human RNaseP gene (as an internal control). All 200 samples amplified the internal control with Cq < 28 and were therefore eligible for further analysis. Over 70% (n = 141) of blood donations were negative for any malaria species. Forty-six donations were positive for both *Plasmodium* targets, while six and seven donations were positive only for *P. falciparum* or *Plasmodium* spp., respectively. All samples (n = 53) with a positive amplification of the Psp18S target were analysed with the species identification assay (PlasID). Apart from confirming *P. falciparum* (n = 33) cases, we also found cases of *P. malariae* (n = 9) and *P. ovale* (n = 2). Amongst the 200 blood samples analysed, no *P. vivax* or *P. knowlesi* was detected.

The assay identified *P. malariae* or *P. ovale* in all seven samples which were positive for *Plasmodium* spp. and negative for *P. falciparum*, highlighting the assays ability to identify non-*falciparum* species. Compared to the PlasQ assay, a reduced sensitivity (71.7%, 33/46) for *P. falciparum* detection using the PlasID assay was observed. The 13 *P. falciparum* positive samples missed by the PlasID assay had moderate parasitaemia (median of 36.9 with IQR of 1.3–380.0 parasites/μL) and the five samples with the highest parasitaemia were positive by RDT. None of these samples were positive for any other non-*P. falciparum* species.

An additional 29 malaria negative blood samples were also run with the PlasID assay to test specificity of the assay. No false positive *Plasmodium* spp. cases were detected, resulting in 100% specificity.

Notably, none of the non-*falciparum* species had been identified by microscopy or RDT. Pan-positive RDTs, which detected the *Plasmodium* spp. LDH, were rather associated with higher *P. falciparum* density than with the detection of non-*falciparum* malaria parasite species. The three HRP2 and LDH positive RDTs had a geometric mean of 916.3 parasites/μL (range 244.1–3707.0) while the nine HRP2 only positive RDTs had a geometric mean of 106.0 parasites/μL (range 11.2–543.3) (Table 2).

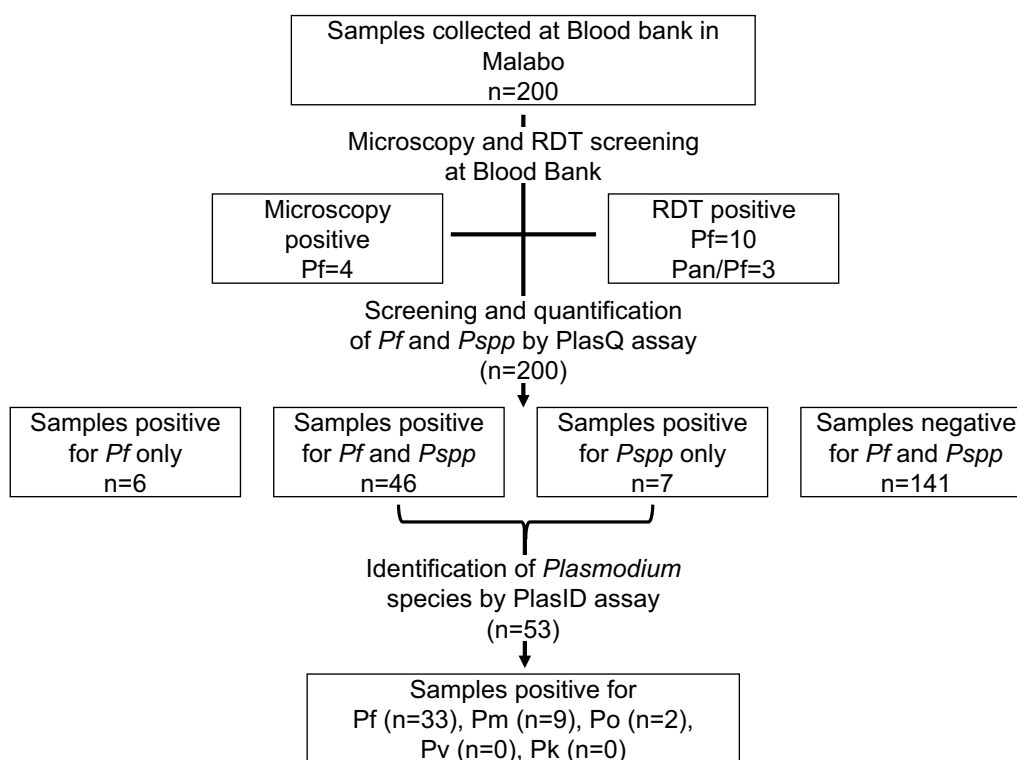
In summary, 59 blood donations were positive for at least one malaria species (29.5%). *Plasmodium falciparum* was the dominating species responsible for 88.1% of positive blood donations, followed by *P. malariae* (15.3%) and *P. ovale* (3.4%). Mixed species infections were found in 6.8% (4/59) of the malaria positive blood donations. One blood donation carried a *P. malariae* and *P. ovale* co-infection and three donations contained

**Table 1 Comparison of health characteristics between voluntary and replacement donors**

	Voluntary donors	Replacement donors	P values <sup>a</sup>
Number of donors	77	123	–
% male	84.4% (65/77)	91.1% (112/123)	0.175
Age in years <sup>b</sup>	32 [19–55]	32 [18–57]	0.888
Weight in kg <sup>b</sup>	73.9 [51–116]	72.0 [52–116.6]	0.593
Blood pressure in mmHg <sup>b</sup>	126.7 [97.7–169.0]	130.8 [103.6–198.9]	0.035
Pulse in bpm <sup>b</sup>	74 [48–101]	73 [51–102]	0.788
Haemoglobin in g/dL <sup>b</sup>	14.6 [12.2–19.6]	14.7 [6.4–18.0]	0.808

<sup>a</sup> Variables were compared by Fisher's exact test for categorical variables or Mann–Whitney test for continuous variables

<sup>b</sup> Values expressed as medians with ranges



**Fig. 2** Overview of blood sample analysis. Blood donations were systematically screened for the presence of malaria parasites by microscopy, RDT and qPCR assays

**Table 2** Parasitaemia of blood donations detected with different diagnostic methods

Diagnostic tool	Median/IQR (parasites/ $\mu$ L) <sup>a</sup>	Geometric mean (parasites/ $\mu$ L) <sup>b</sup>	Range (parasites/ $\mu$ L)
qPCR-PfvarATS	4.6 [0.8–49.0]	5.6 [2.6–12.1]	0.06–3707.0
qPCR-Psp18S	7.8 [1.3–65.1]	9.5 [4.5–19.8]	0.07–3707.0
RDT combined <sup>c</sup>	134.6 [65.1–536.5]	181.7 [68.98–478.7]	11.16–3707.0
HRP2-RDT <sup>d</sup>	112.5 [52.2–331.2]	106.0 [42.4–265.1]	11.16–3707.0
HRP2/LDH-RDT <sup>e</sup>	850.4 [244.1–3707.0]	916.3 [31.11–26990.0]	244.1–3707.0
Microscopy	380.1 [145.4–2909.0]	478.7 [44.03–5205.0]	112.5–3707.0

<sup>a</sup> Quartiles (Median, 25th and 75th percentile)

<sup>b</sup> Geometric mean and 95% confidence interval

<sup>c</sup> Pf- and Pf/Pan-RDT results combined

<sup>d</sup> Pf-RDT results only

<sup>e</sup> Pf/Pan-RDT results only

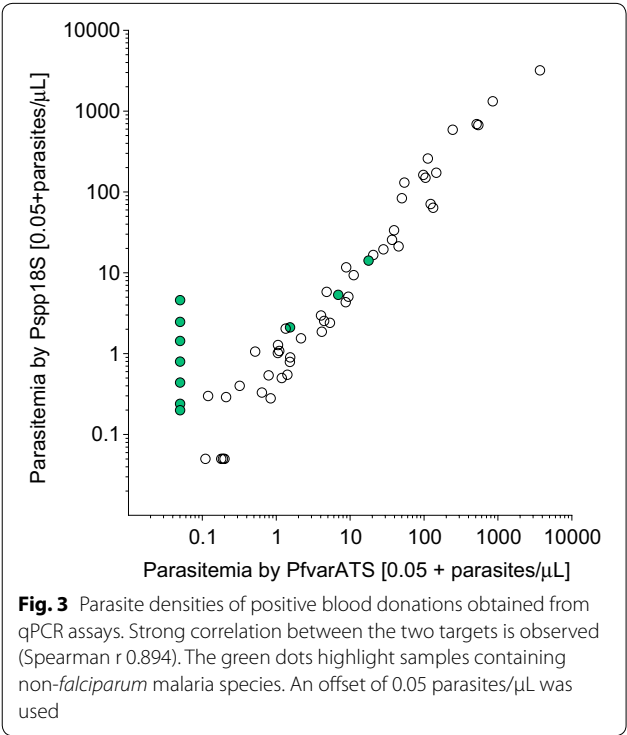
a combination of *P. falciparum* and *P. malariae*. The observed co-infection proportion between *P. falciparum* and *P. malariae* was slightly higher than expected (1.5% versus 1.2%,  $P = 0.037$ ).

#### *Plasmodium falciparum* densities in malaria positive blood donations

Data obtained from the PlasQ assay was used to quantify *P. falciparum* positive blood donations. Parasite densities calculated based on both targets show a high correlation (Spearman  $r = 0.894$ , Fig. 3). Identified

non-*falciparum* species are indicated with green dots. Quantification based on the Psp18S assay revealed, that the parasitaemia of these non-*falciparum* species is around or below 10 parasites/ $\mu$ L which was supported by the high Cq values obtained from the PlasID assay. The geometric mean Cq value of the nine *P. malariae* positive samples was 36.9 (range 35.3–39.0) and the two positive *P. ovale* samples had Cq values of 37.9 and 39.8.

A strong association between the diagnostic tests and the parasitaemia in *P. falciparum* positive blood

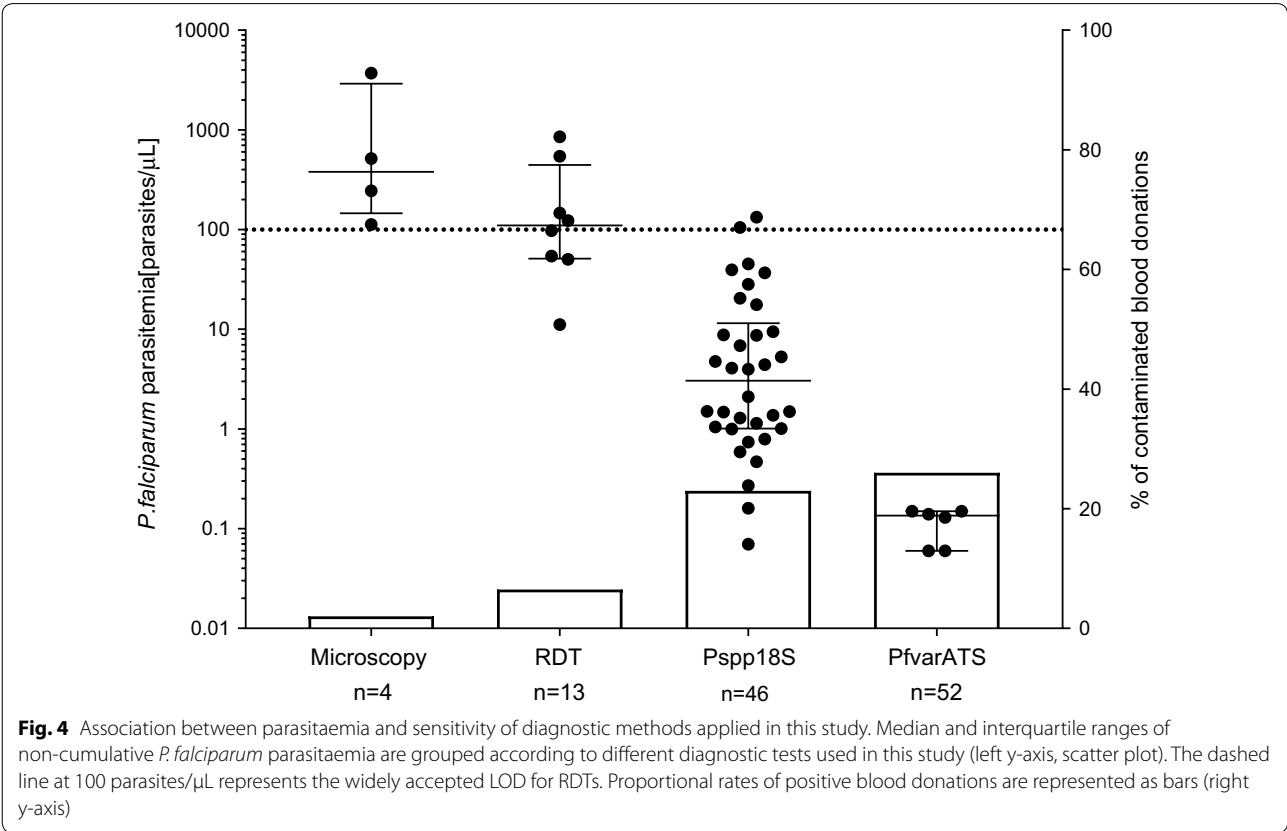


donations was observed. Non-cumulative parasite density data grouped by diagnostic test is shown in Fig. 4 (scatter plot, left y-axis).

In Table 2, the parasite densities grouped by diagnostic tool are summarized. Densities of *P. falciparum* positive blood donations ranged from 0.06 to 3707.0 parasites/ $\mu$ L with a median of 4.6 parasites/ $\mu$ L (IQR 0.8–49.0), which is lower than the LOD for conventional diagnostic tests. Only samples with the highest parasitaemia were detectable by microscopy (381.1 [145.4–2909.0] parasites/ $\mu$ L) and RDTs (134.6 [65.1–536.5] parasites/ $\mu$ L), while the two qPCR assays detected additional infections with lower parasite densities. The median parasitaemia was 7.8 parasites/ $\mu$ L (IQR 1.3–65.1) for the Psp18S target and 4.6 parasites/ $\mu$ L (IQR 0.8–49.0) for the PfvarATS target (Table 2).

**Performance of diagnostic methods for *P. falciparum* detection in blood donations**

In total, 48 previously undetected *P. falciparum* infections were identified by conducting the varATS based qPCR analysis compared to microscopy, increasing the proportion of infected blood donations from 2.0 to



26.0%. The detection rate for RDTs and 18S based qPCR were 6.5% and 23.0%, respectively (Fig. 4, bar plot, right y-axis). This increased detection rate of the qPCR assays is also reflected in analytical sensitivities of the diagnostic tests. Using the PfvarATS qPCR results as the gold standard for *P. falciparum* detection, Pspp18S qPCR (88.5%), RDT (23.1%) and microscopy (7.7%) have all reduced sensitivities. Specificities were for all tests above 95%. One RDT positive sample could not be confirmed by qPCR, while the specificity for Pspp18S was reduced because of non-*falciparum* malaria parasite species, which are also detected (Table 3).

#### Identification of risk factors for malaria positivity

Malaria positive and negative donors were stratified according to the differences in the questionnaire that all blood donors must fill before proceeding to blood donation (Additional file 2). The major risk factor for being a malaria positive blood donor was reporting fever or malaria during the past 3 weeks (Additional file 2). When comparing the anthropometric measurements between malaria positive and negative donors, marginal differences in age and weight become apparent (Table 4). An increased rate of positive blood donations in replacement donors (29.3%) compared to voluntary donors (20.8%) was observed, as well as an increased parasitaemia among replacement donors (8.9 versus 2.1 parasites/ $\mu$ L). However, these differences were not statistically significant.

#### Discussion

This study, conducted in Equatorial Guinea, extends previous observations of presence of TTM in sub-Saharan Africa, and demonstrates the value of NAT approaches to reliably detect sub-patent *P. falciparum* and non-*falciparum* malaria parasites including *P. malariae* and *P. ovale*.

**Table 3 Analytical comparison of methods deployed for *P. falciparum* detection**

Diagnostic tool	Sens <sup>a</sup>	Spec <sup>b</sup>	PPV <sup>c</sup>	NPV <sup>d</sup>
qPCR-PfvarATS	<i>Gold standard</i>			
qPCR-Pspp18S	88.5% [76.6–95.7]	95.3% [90.5–98.1] <sup>e</sup>	86.8% [76.0–93.2] <sup>e</sup>	95.9% [91.7–98.0]
RDT	23.1% [12.5–36.8]	99.3% [96.3–99.9]	92.3% [61.5–100.0]	79.0% [76.0–81.0]
Microscopy	7.7% [2.1–18.5]	100.0% [97.54–100]	100% –	75.5% [74.0–76.9]

<sup>a</sup> Sensitivity and 95% confidence interval

<sup>b</sup> Specificity and 95% confidence interval

<sup>c</sup> Positive predictive value and 95% confidence interval

<sup>d</sup> Negative predictive value and 95% confidence interval

<sup>e</sup> Specificity and PPV of Pspp18S qPCR assay is reduced because non-*falciparum* *Plasmodium* species are also detected

**Table 4 Anthropometric differences in *P. falciparum* positive versus *P. falciparum* negative blood donors**

	Negative donors	Positive donors	P values <sup>a</sup>
Number of donors	148	52	–
% male	86.5% (128/148)	94.2% (49/52)	0.205
Age in years <sup>b</sup>	33 [18–57]	29 [20–55]	0.013
Weight in kg <sup>b</sup>	74.6 [51–116.6]	69.2 [53–110]	0.025
Blood pressure in mmHg <sup>b</sup>	128.7 [97.7–198.9]	128.3 [100.6–156.9]	0.326
Pulse in bpm <sup>b</sup>	74 [48–102]	72 [51–96]	0.519
Haemoglobin in g/dL <sup>b</sup>	14.6 [6.4–19.6]	14.7 [11.6–16.5]	0.319

<sup>a</sup> Fisher's exact test for categorical variables or Mann–Whitney test for continuous variables was applied

<sup>b</sup> Values expressed as medians with ranges

Apart from *P. falciparum*, other malaria causing species such as *P. vivax*, *P. malariae*, and *P. ovale* have been described to circulate on Bioko Island—similar to neighbouring West African countries [27, 28]. Transfusion of any of these malaria species during blood donations have the potential to cause severe disease in recipients. In sub-Saharan Africa, those most at risk are anaemic infants and children, pregnant women, or women who suffer from high blood losses while giving birth [25, 38, 39]. Although, there is a high frequency of TTM in malaria endemic regions, the semi-immunity of recipients may largely control parasite replication and therefore clinical outcome [40].

In malaria non-endemic countries, TTM needs also be taken into account to ensure safe blood donations [41]. Here, blood the recipients are mainly malaria naïve and the immunological control of potentially transfused parasites is non-existent resulting in increased risk of severe malaria disease. *P. falciparum* parasites have been found to survive in blood donations for up to 14 days limiting the potential to neutralize malaria during blood donation processing and storage [42].

This study demonstrates the challenges blood banks in malaria endemic countries encounter. The blood donors at the Malabo blood bank were mainly male adults within an age range of 18–57 years. Because of their pre-existing immunity, blood donors of that age tend to have low levels of parasites without exhibiting clinical symptoms. Ninety-seven percent (97.0%) reported to be in good health at the time of blood donations. Apart from self-reported malaria and/or fever episodes within the last 3 weeks, no other risk factor had been identified based on the provided questionnaire. Since only about 3% (6/198) had reported to have malaria and/or fever episodes, the use of this risk factor to pre-select blood donors would not be very efficient. Deferral of about a quarter of blood



donors that are sub-patent malaria carriers in a region where blood supply shortage is eminent could be even more damaging to public health.

Conventional diagnostic tests are important tools, since they are affordable and can be applied immediately at blood banks, but lack in sensitivity limits their usefulness. Microscopy is still an important tool for malaria detection since it allows for species identification and quantification. The detection limit of an expert microscopy is close to 20–50 parasites/ $\mu$ L, but for non-experienced microscopists, it is up to tenfold higher (i.e. 500 parasites/ $\mu$ L) [5]. Currently available RDTs are based on detection of circulating, parasite derived HRP2, aldolase or lactate dehydrogenase in serum or whole blood samples. These RDTs have the ability to detect *Plasmodium* spp. infections with a LOD between 100 and 1000 parasites/ $\mu$ L which is significantly higher when compared to NATs [43]. In addition, there is currently no single RDT available that could distinguish all five malaria species infections posing a clear limitation to comprehensive malaria infection status monitoring [44, 45]. Infections with *P. malariae* [46], *P. ovale* [47] and *P. vivax* [48] are usually characterized by low parasitaemia levels, rendering these infections largely undetectable by using currently available RDT technologies [49].

In this study, more than three-quarter of *P. falciparum* and all non-*falciparum* infections were missed by the conventional diagnostic tools. A suitable screening method for malaria detection in blood units must have high sensitivity, high negative predictive value, must include all *Plasmodium* species, and should be cost efficient. Such a test must enable a reduction of TTM risk, as well as that of falsely deferred blood donors found to have false positive results.

The PlasQ malaria screening assay applied in this study meets some of these requirements. With a LOD of 0.05 parasites/ $\mu$ L, PlasQ has high sensitivity for *P. falciparum* and includes all other relevant *Plasmodium* species. This assay was extensively used during controlled human malaria infections in Tanzanian and Equatorial Guinean volunteers and no issues with specificity were observed (unpublished). As a limitation, qPCR analysis requires an advanced laboratory infrastructure, trained personnel and the costs for reagents and consumables for this assay is about USD 5, which is higher than the costs for RDT or microscopy-based malaria diagnosis.

This study was used to obtain more information about non-*falciparum* malaria species circulating in Malabo. Therefore a novel multiplex qPCR assay for parallel malaria parasite identification was developed. Using the PlasID assay, the main five human infectious malaria species can be detected qualitatively in a multiplex assay rendering it highly cost efficient since only one DNA

extraction and qPCR reaction needs to be run. This novel malaria species identification assay should be used in combination with the PlasQ assay, based on the finding that in the PlasID assay, a reduced sensitivity for *P. falciparum* is observed. Further development of diagnostic assays for the detection of *P. malariae* and *P. ovale*, should include the ability of species-specific quantification. Estimations based on Psp18S target of the PlasQ assay, indicate that *P. malariae* and *P. ovale* single infections had a geometric mean density of about 0.6 parasites/ $\mu$ L, which is more than 14 times lower than *P. falciparum* single infections (8.8 parasites/ $\mu$ L).

Educating the general public in malaria endemic regions via TV, radio, and pamphlet about the existence of TTM and its potential health impact might help to raise the awareness of this malaria transmission route, both in potential blood donors and medical and nursing staff involved in blood transfusions [50]. This study confirms prior reports that replacement blood donors are particularly prone to harbour sub-patent malaria parasites [51]; hence this sub-population of donors are optimal for such awareness campaigns regarding potential TTM when donating blood for a family or friend. In addition to becoming aware of the potential TTM, medical and nursing staff should also use anti-malarial interventions when the potential for TTM is high and the malaria diagnostic infrastructure is limited. Ultimately, if the goal of malaria elimination is pursued vigorously, tackling TTM in sub-Saharan Africa needs to be included into the malaria elimination agenda.

## Conclusions

More than one quarter of blood donations from healthy donors living in Malabo were infected with malaria parasites. Three species, including *P. falciparum*, *P. malariae* and *P. ovale* were detected using two different qPCR assays. The majority of these infections were not identified by the currently widely used malaria diagnostic tools such as RDTs and microscopy. Since the availability of molecular diagnostic methods in malaria endemic countries is still limited, the blood recipients living in malaria endemic countries should be treated following WHO recommendations.

## Additional files

**Additional file 1.** Oligos used in this study.

**Additional file 2.** Identification of risk factors associated with a malaria positive blood donation.

## Abbreviations

RDT: rapid diagnostic test; HRP2: histidine rich protein 2; LDH: lactate dehydrogenase; LOD: limit of detection; NAT: nucleic acid amplification technology; qPCR: quantitative polymerase chain reaction; TTM: transfusion-transmitted malaria; EGMVI: Equatorial Guinea Malaria Vaccine Initiative; BIMCP: Bioko Island Malaria Control Project; Psp18S: *Pan-Plasmodium* 18S rDNA sequence; PfvarATS: *P. falciparum*-specific acidic terminal sequence of the *var* genes.

## Authors' contributions

Study concept and design: TS, TR, CM, CD. Acquisition of data: TS, TR, JS, LG, LA. Analyses and interpretation of data: TS, TR, MM, CD. Drafting the manuscript: TS, MM, CM, CD and all other authors reviewed the manuscript. Technical or material support: TR, JRB, GG, CC. Study supervision: MM, CM, CD. All authors read and approved the final manuscript.

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## Competing interests

The authors declare that they have no competing interests.

## Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

## Consent for publication

Not applicable.

## Ethics approval and consent to participate

Approval for this study was given by the Ministry of Health and Social Welfare, Malabo, Bioko Norte, Republic of Equatorial Guinea. Study participants were assured of confidentiality and all data collected remained anonymous.

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CASE REPORT

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# Two cases of long-lasting, sub-microscopic *Plasmodium malariae* infections in adults from coastal Tanzania

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## Abstract

**Background:** Malaria is endemic in Tanzania with majority of clinical cases caused by *Plasmodium falciparum*. Additionally, *Plasmodium malariae* and *Plasmodium ovale* spp. are also present and clinical manifestations caused by these infections are not well described. Clinical episodes caused by *P. malariae* infections are often characterized by a relatively mild illness with a low number of parasites, which can persist for long periods. In this report, two cases of *P. malariae* infections that were identified during a clinical trial evaluating the *P. falciparum* malaria vaccine candidate, PfSPZ Vaccine are described. The two participants were followed up and monitored for clinical and laboratory parameters to assess vaccine safety providing the opportunity to study clinical manifestations of *P. malariae* over 4 months.

**Case presentation:** Two young, healthy Tanzanian men infected with low density asexual blood stage *P. malariae* diagnosed by quantitative polymerase chain reaction (qPCR) are described. Retrospective analysis of collected and stored blood samples revealed that the two volunteers had constant asexual blood stage parasitaemia for more than 4 months. During the 132 days of infection, the volunteers' vital signs, body temperature and serum biochemistry all remained within normal ranges. Haematological abnormalities, which were transiently outside normal ranges, were regarded as not clinically significant. During this time period, four consecutive evaluations of blood samples by thick blood smear microscopy conducted by an experienced microscopist were all negative, indicating the presence of low-density sub-microscopic infections.

**Conclusions:** The two cases of *P. malariae* infections presented here confirm the ability of this *Plasmodium* species to persist at low density in the human host for extended time periods without causing clinical symptoms. The presented data also demonstrate that clinical study sites in malaria endemic regions need to have a strong malaria diagnostic infrastructure, including the ability of capturing sub-microscopic parasitaemia and differentiation of *Plasmodium* species.

**Trial registration** ClinicalTrials.gov: NCT02613520, <https://clinicaltrials.gov/ct2/show/NCT02613520>, Registered: November 24th 2015, Enrolment of the first participant to the trial: December 15th 2015, Trial was registered before the first participant was enrolled

**Keywords:** *Plasmodium malariae*, Asymptomatic malaria, Quantitative polymerase chain reaction (qPCR)

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## Background

Malaria is endemic in Tanzania with more than 90% of the population at risk and 5.6 million cases reported by the public health sector in 2017 [1]. While *Plasmodium falciparum* is the dominant malaria species responsible for majority of infections and deaths, other *Plasmodium* species are also endemic in Tanzania. David Clyde, who served as the director of the Malaria Service of the East African Malaria Institute at Amani, described the occurrence of four human malaria species, including *P. falciparum*, *Plasmodium vivax*, *Plasmodium ovale* spp. and *Plasmodium malariae* in his 1967 book “*Malaria in Tanzania*” [2]. *Plasmodium vivax* was attributed to importation by Indian immigrants during the first world war and since 1917 this influx has virtually ceased. *Plasmodium malariae* was observed in 10–20% of malaria infections, mainly as co-infections with *P. falciparum* and during childhood [2]. More recently, a microscopy-based cross-sectional survey conducted in the Tanga region of coastal Tanzania found very few infections with *P. malariae* (0.3%) or *P. ovale* spp. (0.1%) [3]. Data collected in coastal Tanzania, confirm these low numbers of non-*P. falciparum* *Plasmodium* infections. Diagnosis by qPCR revealed low prevalence for *P. malariae* (0.7–5.8%) and *P. ovale* spp. (0.9–1.1%) among asymptomatic school children (Schindler et al., unpublished data). Since microscopic diagnosis of *P. malariae* asexual blood stage parasites is hampered by the low parasitaemia and morphological similarities to *P. falciparum*, molecular based, highly sensitive diagnostic methods are needed to establish the true prevalence of this parasite in the population [4]. Lack of sensitive *P. malariae* diagnosis methods applicable in the field and the research focus on *P. falciparum* has resulted in significant knowledge gaps regarding spectrum of potential clinical manifestations and burden of *P. malariae* infections [5].

It is well established that *P. malariae* is widespread throughout sub-Saharan Africa, South East Asia and Latin America and the biology of *P. malariae* was reviewed by Collins et al. [6]. Treatment of syphilis by controlled infections with *P. malariae* provided valuable insight into human-parasite interactions. The red blood cell cycle lasts 72 h with an average of 8 merozoites released per schizont and the parasite prefers to infect and develop in older erythrocytes. So far, no evidence for a dormant liver stage as described in *P. vivax* and *P. ovale* spp. has been observed. Faster acquisition of immunity against *P. malariae* compared to immune responses against *P. falciparum* has been described [6].

Clinical episodes of *P. malariae* infections are characterized by a mild illness caused by low numbers of parasites which can persist for extremely long periods, often for years or even decades [6]. There are reports of cases

of *P. malariae* caused illness from Greece [7] and Trinidad and Tobago [8] decades after eradication of malaria from these regions. Chronic *P. malariae* infections have been considered a major cause of the nephrotic syndrome in the past, although the incidence of *P. malariae*-associated nephrotic syndrome has been dramatically reduced in recent decades [7–9]. Recently, it was demonstrated that the controlled infection of two volunteers with cryopreserved *P. malariae*-infected erythrocytes was well tolerated and no severe or serious adverse effect, or biochemical abnormalities were observed [10].

The clinical research facility of the Ifakara Health Institute in Bagamoyo, Tanzania, conducts clinical trials evaluating efficacy of experimental malaria vaccines in the target population [11–13]. A controlled human malaria infection (CHMI) model has been successfully established since 2012 [14]. As part of these clinical trials, participants are closely monitored to identify any abnormal clinical or laboratory parameters in order to evaluate vaccine safety and tolerability. Regularly, volunteers are screened for *Plasmodium* spp. parasites in blood using thick blood smear microscopy as well as quantitative polymerase chain reaction (qPCR). The volunteers described in this report participated in a study evaluating the safety and efficacy of immunization with Sanaria® PfSPZ Vaccine composed of radiation attenuated, aseptic, purified, cryopreserved *P. falciparum* sporozoites (PfSPZ) [11, 15–19] which was conducted between 2015 and 2016 (clinicaltrials.gov: NCT02613520) [20]. The clinical cases of two young men infected with asexual blood stage *P. malariae* as diagnosed by qPCR are described. These volunteers were followed closely for 4 months during the clinical trial.

## Case presentation

Two male residents of Bagamoyo, 20 and 22 years of age, were enrolled into the clinical trial based on predefined exclusion and inclusion criteria as outlined in the clinical trial protocol. A review of the medical history, physical examination, vital signs (pulse, blood pressure, and respiratory rate), and ECG did not reveal any abnormalities. At screening, the volunteers had negative serologies for human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV). A single stool sample collected at study enrolment was negative for intestinal helminths and no *Schistosoma haematobium* eggs were detected in urine. No blood biochemistry abnormalities were detected, which included alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (BIL), and creatinine (CRE). The urine analysis using a 13-parameter dipstick (Combinia 13 test strips, HUMAN Diagnostics, Germany) was negative at enrolment. A complete blood count (CBC) was

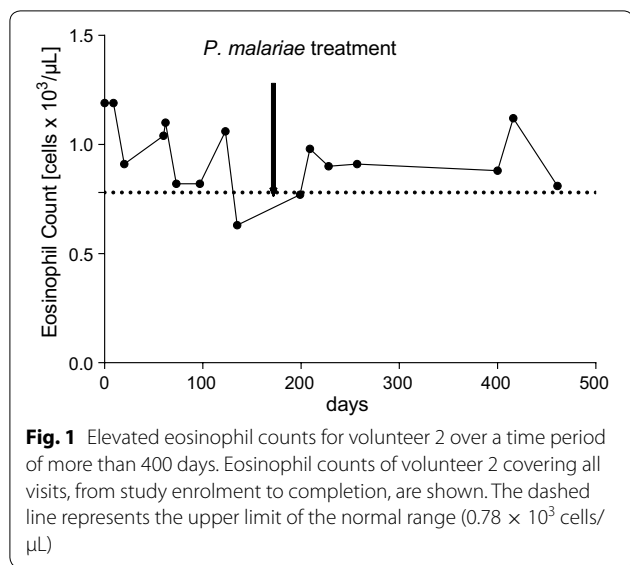
**Table 1 Overview of clinical and parasitological parameters assessed for volunteer 1**

	Study enrolment	Routine visit 1	Routine visit 2	qPCR results reported	Post-treatment visit 1	Post-treatment visit 2
Timeline						
Date	22/12/2015	13/02/2016	13/04/2016	28/04/2016	06/05/2016	01/07/2016
Days respective to enrolment into clinical trial	0	53	113	128	136	192
Clinical evaluation						
Body temperature	36.6 °C	36.2 °C	36.8 °C	36.6 °C	–	37.2 °C
Vital signs including pulse, blood pressure and respiration rate	NAD	NAD	NAD	NAD	–	NAD
Laboratory evaluation						
Biochemistry including ALT, AST	NAD, also included BIL and CRE at enrolment	NAD	NAD	NAD	–	NAD
Complete blood count includes RBC, HGB, HCT, MCV, MCH, MCHC, PLT, WBC with differential	NAD	NEUT ↑ (3.57 × 10 <sup>3</sup> /μL, 72.2%) LYMP ↓ (0.70 × 10 <sup>3</sup> /μL, 14.1%)	MCHC ↓ (29.7 g/dL)	NAD	–	NAD
Urine analysis using dipstick that includes bilirubin, ketone, creatinine, hematuria, proteinuria, albumin, nitrite, leucocytes, glucose, specific gravity, pH, vitamin C	NAD	NAD	Proteinuria (1+)	NAD	–	NAD
Serology (HIV, HBV, HCV)	Negative	–	–	–	–	–
Screening for intestinal helminths and schistosomiasis	Negative	–	–	–	–	–
Malaria diagnostics						
Thick blood smear microscopy	Negative	Negative	Negative	Negative	Negative	Negative
Screening and species identification qPCR assays	Screening qPCR: <i>Cq</i> of 36.03 ID-qPCR: <i>P. malariae</i>	Screening qPCR: <i>Cq</i> of 35.72 ID-qPCR: <i>P. malariae</i>	Screening qPCR: <i>Cq</i> of 37.69 ID-qPCR: <i>neg</i> <sup>a</sup>	Screening qPCR: <i>Cq</i> of 34.47 ID-qPCR: <i>P. malariae</i>	Screening qPCR: <i>neg</i>	Screening qPCR: <i>neg</i>
Drug treatment						
Prescription of drugs throughout the clinical trial	–	–	–	Start of artesunate-amodiaquine based treatment on 02/05/2016	–	–

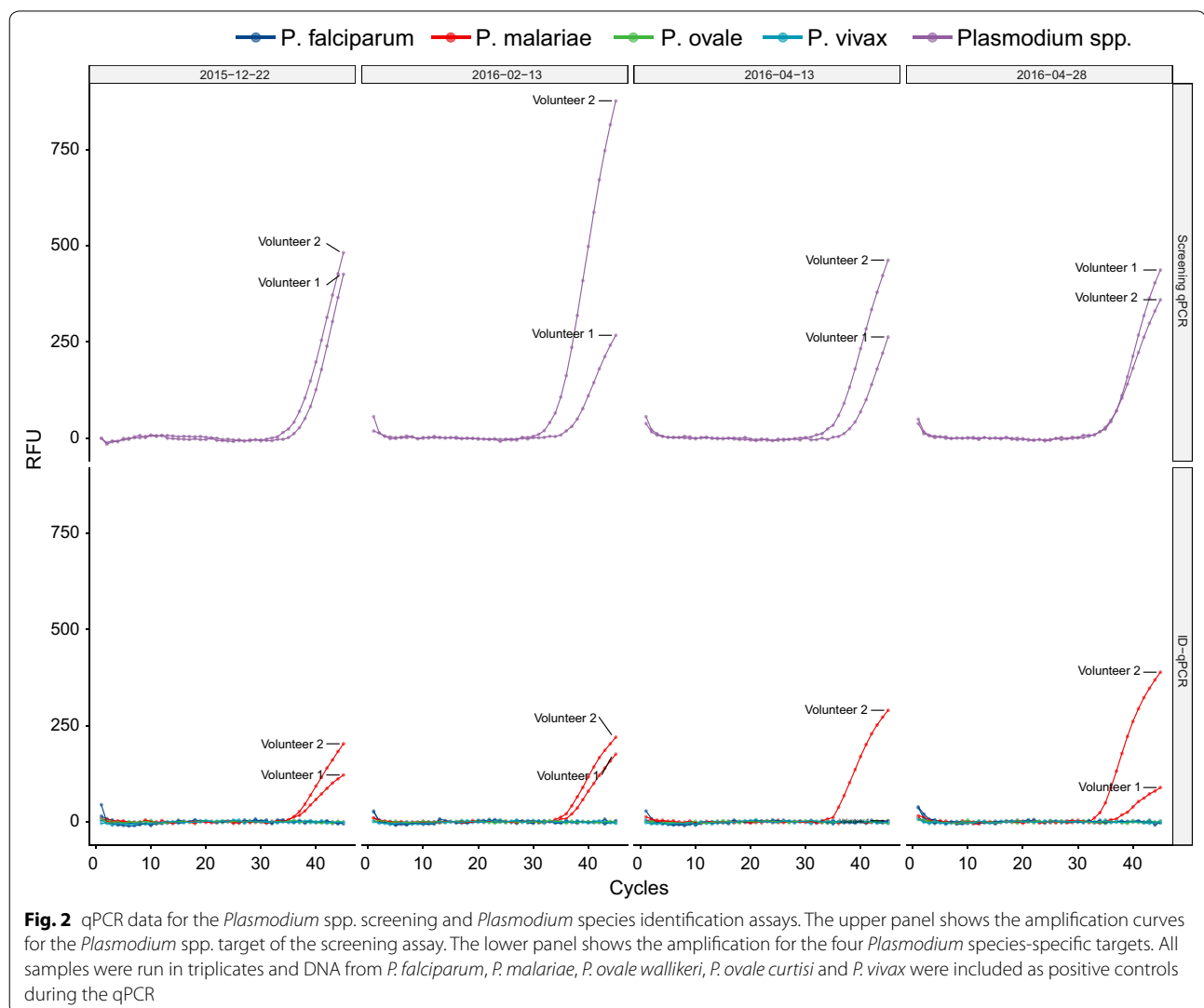
NAD no abnormalities detected

<sup>a</sup> *Plasmodium* species identification qPCR assay was negative due to drop of *P. malariae* parasitemia (Cq value for 18S *Plasmodium* spp. screening assay was 37.69)





conducted at screening and showed normal haematological parameters for volunteer 1, while volunteer 2 had elevated eosinophil counts ( $1.19 \times 10^3/\mu\text{L}$  compared to the pre-defined upper normal range of  $0.78 \times 10^3$  cells/ $\mu\text{L}$ ). During the 16 weeks of immunization (3 doses of PfSPZ Vaccine at 8-week intervals), the volunteers' health status was closely monitored. Every 8 weeks at pre-defined visits blood was drawn and any deviations from reference laboratory values were reported. Vital signs, body temperature and biochemistry remained within normal ranges when assessed four times during the follow up period. Haematological parameters which were transiently outside normal ranges in both volunteers were regarded as not clinically significant (Tables 1, 2). Noteworthy, for volunteer 2 the eosinophil counts remained consistently elevated until the end of the study (Fig. 1). Both volunteers tested once positive for low grade proteinuria, during the study by urine dipstick (Tables 1, 2).



After concluding three PfSPZ Vaccine immunizations, and before vaccine efficacy was assessed by CHMI, the study protocol required screening of whole blood by qPCR to detect sub-microscopic malaria parasitaemia, so that these volunteers could be treated accordingly before participation in CHMI. During this routine visit, qPCR was conducted with fresh blood samples and it was discovered that two volunteers were infected with *Plasmodium* spp. parasites [21]. Based on *P. malariae* species-specific qPCR [22] and conventional nested PCR [23], *P. malariae* infections were confirmed. The presence of *P. falciparum* [24, 25], *P. ovale* spp. [26], and *P. vivax* [25] was excluded by qPCR and conventional PCR [23].

Treatment with 3 doses of artesunate/amodiaquine (200/540 mg) daily for 3 days was initiated, and complete parasite clearance was confirmed by qPCR 4 days later. Both volunteers then underwent CHMI and remained in the clinical trial until study completion. Within the following 296 days until the completion of the clinical trial, no recurrent (recrudescence or new infection) *P. malariae* parasitaemia was observed. Both volunteers were negative for *P. falciparum* after the first CHMI, and both became positive for *P. falciparum* after a second CHMI at 40 weeks after the last immunization and were successfully treated with artemether/lumefantrine.

Blood samples collected during the clinical trial and stored frozen were analysed retrospectively by qPCR to determine the time point of *P. malariae* infection. It turned out that both volunteers had *P. malariae* parasitaemia at enrolment into the clinical trial. Both volunteers remained positive throughout the vaccination period. *Plasmodium malariae* parasites were detectable at four out of four clinical visits, namely at day 0, 53, 113 and 128 of study and malaria treatment took place 132 days after the first detection of the *P. malariae* infections (Fig. 2). Evaluation of four blood samples collected at the same days by thick blood smear microscopy and conducted by an experienced microscopist was reported as negative. Thick blood smear preparation and reading was performed according to our standard operating procedure followed during CHMI studies [14]. The negative microscopy results and the high Cq values (median of 34.1 with a range of 31.6–37.7) obtained by the *Plasmodium* spp. qPCR assay indicate that the parasitaemia levels were low. When compared to qPCR based detection of *P. falciparum* 18S gene, these Cq values would correspond to a parasitaemia between 1 and 10 *P. malariae* parasites per  $\mu$ L blood [27].

## Discussion and conclusion

The two *P. malariae* cases presented here confirm the ability of this *Plasmodium* species to persist at low density in the human host for extended time periods without

causing clinical symptoms or signs. Both were detected in clinically healthy, young men participating in a clinical trial of PfSPZ Vaccine. No abnormalities in vital signs, alanine aminotransferase, aspartate aminotransferase, total bilirubin, and creatinine serum levels were detected. Except for a one-time low-level proteinuria, urine analysis parameters measured by dipstick remained within physiological ranges and there was no indication of impaired renal function in these two volunteers. Volunteer 2 did have mildly elevated eosinophil counts throughout the entire course of the clinical trial. These levels were not affected by the treatment of the *P. malariae* infection and may have reflected an ongoing intestinal helminth infection that was too low to be detected by a single stool examination. All other haematological abnormalities were of temporary nature and considered to be not clinically significant. Interestingly, the *P. malariae* parasitaemia levels were not affected by the three rounds of PfSPZ Vaccine immunizations. This might be due the mode-of-action of the vaccine which is thought to act against the liver-stage of the parasite.

The data presented in this report demonstrates that study sites in malaria endemic regions conducting clinical trials should develop on site malaria diagnostic infrastructure, which includes the detection of low-density asexual blood stage parasitaemia and identification of different *Plasmodium* species. Eventually, if the goal of malaria elimination is pursued vigorously, the implementation of highly sensitive diagnostic methods to detect asymptomatic, low-density *P. malariae* infections need to be included into the malaria elimination agenda.

## Abbreviations

PfSPZ: *P. falciparum* sporozoites; CHMI: controlled human malaria infections; HIV: human immunodeficiency virus; HBV: hepatitis B virus; HCV: hepatitis C virus; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BIL: total bilirubin; CRE: creatinine; CBC: complete blood count; RBC: red blood cells; HGB: haemoglobin; HCT: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; PLT: platelets; WBC: white blood cells; NEUT: neutrophils; LYMP: lymphocytes; EO: eosinophils; RDW-SD: red cell distribution width; TBS: thick blood smear; qPCR: quantitative polymerase chain reaction; Cq: quantification cycles of qPCR reaction; ID-qPCR: *Plasmodium* species identification qPCR assay; NAD: no abnormalities detected.

## Authors' contributions

SJ, MT, SLH, SA and CD designed the study. TS, MM, GM, SM and JS conducted the laboratory analysis. SJ, FS, KR, LWPC and TLR collected and analysed the clinical data. TS, SJ, SLH, LWPC, TLR and CD interpreted the findings and wrote the manuscript. All authors read and approved the final manuscript.

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## Competing interests

LWP Church, TL Richie and SL Hoffman are salaried, full time employees of Sanaria Inc, the developer and sponsor of Sanaria® PfSPZ Vaccine. These individuals were not responsible for the collection, recording or entry of the clinical and parasitological data presented here. The other authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in the subject matter or materials discussed in the manuscript.

## Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

## Consent for publication

Written informed consent was granted from the patients for publication of clinical and laboratory data obtained during the clinical trial on condition of anonymity.

## Ethics approval and consent to participate

The two volunteers described in this case report consented to participate in a clinical study, registered at ClinicalTrials.gov (NCT02613520), and conducted under a U.S. FDA IND application. The study was performed in accordance with Good Clinical Practices. All data presented in this case report were obtained according to the approved study protocol. The protocol was approved by the institutional review boards of the Ifakara Health Institute (Ref. No. IHI/IRB/129 No: 32-2015), and the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/2049), by the Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland (reference number 15/104), and by the Tanzania Food and Drug Authority (Auth. No. TZ15CT013).

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# Chapter V

## Discussion



## 5.1 Evaluation of the PfSPZ Vaccine in two different malaria pre-exposed populations

An effective vaccine against malaria would contribute enormously to global health. The development of vaccines against complex and chronic infectious diseases like HIV, tuberculosis and malaria has proven to be challenging [130]. It has become apparent that among the biggest challenge for successful vaccine development is the fact that different populations show variations in immune responses to the same vaccine [131]. Therefore, in order to prevent failure of an experimental vaccine, candidates should be evaluated early on during the development process in the major target population – best by a human challenge model.

The PfSPZ Vaccine, a malaria vaccine based on radiation-attenuated sporozoites, has proven to be safe and well tolerated in target populations including infants, children, adolescent, adults and people living with HIV infection. Compared to malaria-naïve US Americans [126], the VE of PfSPZ Vaccine was significantly lower in Tanzanian [124] and Malian vaccinees [94] using an identical immunization regimen. Among the US Americans the VE was 92% (12/13 protected) assessed by homologous mosquito-bite CHMI, in Tanzania the VE was 20% (4/20 protected) against homologous CHMI using PfSPZ Challenge and in Mali the VE was found to be 29% in a field trial against heterologous natural *P. falciparum* infections. Even by increasing the dosage of RAS, the protection rates as seen in malaria-naïve volunteers were never achieved in the PfSPZ Vaccine trials in Tanzania and Equatorial Guinea. The same was observed for the PfSPZ-CVac approach, while the immunization with three doses of  $5.12 \times 10^4$  sporozoites under chloroquine coverage was 100% effective (9/9 protected) in malaria naïve vaccinees, even higher doses of three times  $1.0 \times 10^5$  did not nearly have comparable VE in Equatorial Guinea, where efficacy was calculated to be 57% (8/14 protected).

A generally weaker responsiveness to a number of vaccines in developing countries has been known for a quite a while [132]. Several vaccination studies have shown that children from Sub-Saharan Africa are less responsive to standard childhood vaccines than children from developed countries [133]. This was shown for vaccines against tuberculosis, typhoid fever, measles and polio vaccines (summarized in [133]). The underlying mechanisms are complex and multifactorial at best and the reason for this variation might be found in the host-environment interaction [131]. In general, heterogeneity in vaccine responsiveness has been shown to be influenced by age [134], gender [135], genetics [136, 137], nutritional status [138] and has also been associated with the microbiome [139]. Co-infections with helminths, protozoans and viruses can result in decreased vaccine efficacy [133]. Especially, chronic immune activation, as induced by viral or helminth infections, has been associated with reduced immune responsiveness [140–142]. Deworming communities has shown to reduce the helminth-induced immune hypo-responsiveness against vaccines [143].

## **5.2 The use of CHMI for assessing vaccine efficacy**

The use of CHMI to assess vaccine efficacy in malaria pre-exposed individuals has been proven to provide protection data that fast tracks identification of best vaccine dosing and regimen. Compared to large field studies, CHMI studies allow to test various vaccine regimens and doses in relatively small groups in a shorter time frame. A simple way to demonstrate how effective CHMI is in evaluating malaria vaccines, is to look at the data from the six clinical trials described in this thesis. Two different attenuation procedures (RAS and PfSPZ-CVac), various vaccination regimens, administering all immunization within 6 months to within 1 week, and 8 different doses of total RAS ranging from  $6.75 \times 10^5$  to  $8.1 \times 10^6$ ,

have been evaluated. All this was achieved in less than five years at two clinical trials sites with just 197 enrolled and vaccinated volunteers. In the same time, other clinical trial facilities located in Kenya, Gabon and Mali also conducted CHMIs to evaluate the PfSPZ Vaccine and to understand host-pathogen interaction under defined conditions.

CHMI studies are a valuable tool for dose finding and assessing immunogenicity, during the early phase of clinical development of vaccines, but by using relatively small numbers of volunteers the ability to predict effectiveness of a vaccine in a larger population might be limited. Clearly, CHMI can only be conducted in adult volunteers and the vaccine induced immune responses in infants and children might differ. An additional limitation is that only four *P. falciparum* strains are currently in use for CHMI studies [107], which does not necessarily reflect the global range of genetic diversity of *P. falciparum* circulating in endemic regions. This is highly relevant since during the RTS,S/AS01 phase 3 trial, it was found that the vaccine effectiveness was impacted by the genetic diversity of the parasite [144]. Given these limitations, larger scale field trials assessing the effectiveness of vaccines in the target populations can hardly be replaced by CHMI studies.

Besides testing the efficacy of vaccines, conducting CHMIs in developing countries offer also a great opportunity for capacity building and implementation of new techniques. We used the molecular malaria diagnosis platform for capacity building including training of personnel in Tanzania and Equatorial Guinea. Additionally, CHMIs also serve as a biobank for biological samples which can be used to evaluate and compare novel diagnostic methods using highly standardized blood samples with known parasite strains present.

### 5.3 The impact of naturally acquired immunity on vaccination and CHMI

The principal features of NAI, the ability to control parasite density and disease, have been defined, but little is known about the underlying immune effector mechanisms [46]. Epidemiological data show the widespread and persistence of parasitaemia in asymptomatic individuals and that natural exposure to sporozoites does not induce complete, sterile immunity [145] indicate that NAI is predominantly directed against the blood-stage of the parasite [46]. How could then NAI impact the vaccination outcomes with RAS as we have seen in volunteers from endemic regions? One observation made during the BSPZV1 study, where higher levels of memory CD4 and CD8 T cells at baseline in the Tanzanian compared to the US volunteers were seen, could provide some explanation. Less available naïve cells for expansion during the vaccinations might explain the lower immunogenicity [124]. The interaction between NAI and vaccine take and responsiveness of PfSPZ Vaccine remains subject of debate and future research.

The impact of NAI on parasite growth dynamics during CHMI has been described previously. The PMR, the fold change in number of parasites in the blood over one life cycle, can be calculated based on the qPCR-derived parasite densities measured during the pre-patent period [146]. Using CHMI to infect malaria-naïve volunteers found an average PMR of about 10 [147], while if the same CHMI is conducted in volunteers living in malaria endemic regions - PMRs are significantly lower and more variable [113, 116, 148, 149]. We used two different approaches to demonstrate the impact of recent malaria pre-exposure on the multiplication rate of *P. falciparum* parasites. We showed that recently cleared parasitaemia from an ongoing *P. falciparum* single infection or *P. falciparum* and *P. malariae* co-infection leads to a significantly reduced PMR. And secondly, that the PMR is significantly reduced after a second homologous CHMI conducted in the identical volunteer. This data indicates the devel-

opment of parasite growth reducing immune effector mechanisms during a single episode of asexual blood stage infection. The biological specimens collected from these volunteers before, during and after CHMI will help to further identify and dissect these immune effector mechanisms. Additionally, the data presented here on interaction between HIV and *P. falciparum* demonstrate the ability of CHMI to provide the framework to study the impact of chronic co-infections on host-pathogen interaction.

## 5.4 Development and implementation of tools for molecular malaria surveillance

We used these vaccine trials to establish a molecular diagnostic platform on-site in two malaria endemic countries. As a next step, we wanted to advance these techniques for use in the context of a broader approach – larger scale molecular surveillance of malaria. We addressed several key features, which in our opinion are critical to successfully run a molecular diagnostic platform. First, robust qPCR assays for quantification of *Plasmodium* spp. parasites should be used, which includes the establishment of a local supply chain for consumables and reagents. Second, a program for laboratory-based training and qualification of local personnel should be started early on. Lastly, user-friendly software solutions, e.g. ELIMU-MDx, will not only reduce time spent on data cleaning and analysis, but also increase quality and integrity of qPCR data.

*P. falciparum* strains carrying *pfhrp2/3* deletions and evading detection by RDTs pose a threat to malaria control and elimination programs around the world [42]. Together with the emergence of artemisinin resistant strains, these parasites are a powerful example that molecular surveillance systems need to be implemented in malaria control programs to monitor their potential expansion over time. A central part of molecular surveillance systems are novel NAT-based assays enabling high-throughput screening of *P. falciparum* populations to identify isolates threatening malaria control efforts. As for the identification of *pfhrp2/3* deletions, the currently used methods, mostly based on nested PCR, are extremely time consuming, and therefore impractical for screening of large number of samples, as required for a country-wide survey of *pfhrp2/3* deletions. With growing availability of qPCR instruments in reference laboratories in Sub-Saharan countries, our novel assay could be used as a surveillance tool to monitor over time the potential expansion of *P. falciparum* strains carrying *pfhrp2/3* deletions.

Speciation of *Plasmodium* spp. parasites is another application of molecular diagnostic tools for surveillance of malaria. In Sub-Saharan Africa, *P. malariae* and *P. ovale* spp. are rarely detected by conventional diagnostic methods. The inability of detecting these parasites and with *P. falciparum* dominating the research agenda, has left the malaria research community with a knowledge gap concerning the health burden of chronic *P. malariae* or re-occurring and relapsing *P. ovale* spp. infections in an endemic context [23]. Most importantly, the question on how *P. malariae* and *P. ovale* spp. interact with *P. falciparum* in co-infected individuals can only be addressed if sensitive and specific diagnostic assays are used [23]. Several studies indicate that a mixed infection with *P. malariae* may reduce *P. falciparum* caused symptoms [150–152]. On the contrary, a study conducted in the Gambia observed more *P. malariae* clinical episodes during the dry season, when *P. falciparum* infections are less common, concluding that *P. falciparum* may reduce *P. malariae* infection related symptoms [153]. Understanding these interactions is of highest importance, if the introduction of species-specific intervention tool, such as a *P. falciparum* vaccine, is considered. How are the infection dynamics and clinical features of *P. malariae* and *P. ovale* spp. infections impacted in a certain epidemiological context, if we specifically and exclusively target *P. falciparum* by a vaccine?

Molecular malaria surveillance should be closely integrated into the existing health systems. We used two different platforms to evaluate NAT-based assays for malaria surveillance. First, we collaborated with the central blood bank in Malabo, Equatorial Guinea. The access to fresh and highly abundant blood makes blood banks a useful source of samples for disease surveillance. On the contrary, populations donating blood are often not very heterogeneous and do not reflect the general population. Our second approach included the use of RDTs collected during the annual malaria indicator survey (MIS) on Biko Island, Equatorial Guinea during 2018. Using the small amount of blood found on RDTs is a convenient and cost-

effective procedure which would reduce phlebotomy and blood collection to a minimum which is beneficial to the volunteer participation. Here, we addressed the steps needed to establish molecular disease surveillance program depending on the analysis of nucleic acids extracted from RDTs. During the MIS on Bioko Island in 2018, 13'505 people gave consent to the molecular analysis of their RDTs, which is currently ongoing. And with the extraction of nucleic acids from RDTs (ENAR) protocol, we developed a procedure that allows to extract nucleic acids from RDTs in a high-throughput manner.



# Chapter VI

## Conclusion and future directions

More than one decade after a renewed scientific and political commitment to malaria eradication [154, 155], the WHO reports an alarming finding that no further significant reduction of global malaria cases was observed between 2015-2017 [12]. Despite these setbacks, in April 2019, the first children in Malawi, Ghana and soon also in Kenya, were enrolled into a malaria vaccine implementation program (MVIP), coordinated by WHO, to assess the feasibility of administering the required 4 doses of RTS,S /AS01 [79].

Other vaccine candidates have reached different phases of clinical development. Among one of the most advanced vaccines is the PfSPZ Vaccine, which is based on the immunization with radiation-attenuated sporozoites. This vaccination approach has shown to be highly effective in malaria-naïve volunteers but failed to reproduce the same degree of protection in different malaria pre-exposed populations. The reason for lower protection in these vaccinees remains subject to further studies. As for now, optimization of the vaccine regimen in the target population might be the key to better vaccine efficacy. Increasing the total dosage of RAS did show some improvement in protection against homologous CHMI in Tanzanian volunteers. In Equatorial Guinea, a study comparing different regimens of PfSPZ Vaccine, focusing on short intervals between the immunizations, was recently completed and the data is currently analysed (EGSPZV3 study). Alternative attenuation procedures of the sporozoites also show promising results in human studies. Immunizations with sporozoites under chloroquine coverage did result in higher vaccine efficacy compared to immunization with RAS in a side-by-side comparison in Equatorial Guinean adults (manuscript in preparation). Genetically attenuated parasites (GAP), which arrest their development during liver-stage due to targeted gene deletions, have recently transitioned into the early clinical phase [89]. No GAP has been tested for efficacy by CHMI or in an endemic setting for protection against field isolates so far.

In order to better identify mechanism of protection associated with PfSPZ vaccination, getting access to liver biopsies for analysing liver-specific transcriptome and immunological changes upon vaccination would be desirable. Similar experiments were conducted successfully to study chronic hepatitis B infections [156]. At the Jenner Institute in Oxford, UK, liver cell aspirates have been taken from volunteers undergoing prime target vaccination approaches and the results will inform future research along these lines (unpublished)

CHMI is increasingly used in malaria endemic regions, where it has been shown to be safe, including in vulnerable populations such as in immunocompromised people living with HIV. Vaccine efficacy can be assessed rapidly and in small groups directly in the target population. Based on our experience, one of the major inconveniences for the volunteers participating in CHMI is the time they have to spend at the clinical trial facility, which can take up to two weeks and also includes over-night stays. The same issue has also been raised by our colleagues in Kenya [123, 157]. By changing the primary outcome from TBS microscopy to qPCR positivity as proposed for CHMIs in non-endemic regions [118], the time spend in the clinical trial facility could be significantly decreased. This is especially important for populations where approximately 10% stay sub-microscopic for asexual blood stage parasitaemia and qPCR is detecting parasites on average 5.5 days earlier than microscopy. Not changing the primary endpoint of a CHMI to qPCR does unnecessarily increase the duration of CHMIs for studies assessing efficacy of pre-erythrocytic vaccines. Looking ahead, further development of the CHMI facilities in Bagamoyo and Malabo should include the evaluation of other *P. falciparum* strains apart from PfNF54. Testing vaccine efficacy against different heterologous strains would be in line with recommendations of the US FDA [107]. Extending the CHMI platform to blood-stage challenge with non-*falciparum Plasmodium* species, as recently shown with *P. malariae* [102], would be desirable. This would provide insight into the interaction of different malaria species in co-infections. Also, the immune responses targeting

the different *Plasmodium* spp. species in the target population becomes amenable for in depth studies.

As useful as CHMIs are during early phases of clinical development of a vaccine, it might not be suitable to replace large phase 3 field trials evaluating the effectiveness of PfSPZ Vaccine in the population. Currently, the planning for a phase 3 trial to evaluate PfSPZ Vaccine on Bioko Island is ongoing. The vaccine efficacy will be determined in a double-blind controlled study involving 700 placebos and 1400 vaccinees in the age category of 2 years to 45 years of age. After this study, a cluster randomized PfSPZ vaccine trial is planned that will inform about the development of herd immunity induced by PfSPZ Vaccine against malaria. The use of molecular diagnostic tools will be critical as the sensitivity of TBS microscopy or RDTs is not sufficient to identify the majority of *P. falciparum* infections in the Equato-Guinean population. It will be of greatest interest to see if failure in vaccine protection will be associated with certain *P. falciparum* genotypes. We propose to collect samples to conduct a sieve analysis in order to identify genotypes evading the PfSPZ Vaccine [144]. The molecular surveillance during the phase 3 trial should be extended to other relevant pathogens which might act as confounder for vaccine responsiveness and vaccine induced protection. We have observed a high prevalence of filarial nematodes on Bioko Island, in particular *Loa loa* and *Mansonella perstans*, which are well known for their immunomodulatory properties impacting potentially negatively on vaccination outcomes [158–160].

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# Appendix

Appendix A. *Curriculum vitae* Tobias Schindler

Appendix B. Publication list Tobias Schindler

# CURRICULUM VITAE - TOBIAS SCHINDLER

Date of birth: 17/03/1987  
Nationality: Swiss  
Address: Grubenstrasse 27, CH-4142 Münchenstein, Switzerland  
Phone: +41 79 256 41 02  
E-mail: schindler.tobi@gmail.com

## EDUCATION

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07/2019	<b>PhD in Epidemiology</b>	<i>University of Basel</i>
01/2015	<b>Master of Science in Molecular Biology</b>	<i>University of Basel</i>
07/2011	<b>Bachelor of Science in Biology</b>	<i>University of Basel</i>
07/2009	<b>Bachelor of Science in Molecular Life Sciences</b>	<i>University of Applied Sciences FHNW</i>
08/2006	<b>Swiss Certificate of Competence as a laboratory technician</b>	<i>Novartis Pharma AG</i>

## WORK EXPERIENCE

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05/2015 – 07/2019	<b>PhD project</b>	<i>Swiss Tropical and Public Health Institute</i>
	Thesis: Developing, testing and implementing novel molecular diagnostic tests and procedures for malaria at the individual and population level in East and West Africa	
01/2015 – 04/2015	<b>Research assistant</b>	<i>Swiss Tropical and Public Health Institute</i>
	Laboratory analysis and supply chain management within the framework of phase I vaccine trials in Tanzania and Equatorial Guinea.	
09/2013 – 12/2014	<b>Master thesis project in industry</b>	<i>F. Hoffmann-La Roche AG</i>
	Master thesis project was conducted in collaboration with the Department of Translational Technologies and Bioinformatics.	
07/2011 – 08/2013	<b>Civilian service in Tanzania</b>	<i>Swiss Tropical and Public Health Institute</i>
	Support of epidemiological studies, assessing the co-morbidity of helminth and malaria infections in children, conducted at the Ifakara Health Institute in Bagamoyo, Tanzania.	

01/2009 – 02/2011	<b>Part-time job as laboratory technician</b>	<i>Biolytix AG</i>
	Routine laboratory analysis in microbiological water, feed and food analysis.	
08/2003 - 08/2006	<b>Apprenticeship as a laboratory technician</b>	<i>Novartis Pharma AG</i>
	Extensive training in basic laboratory methodologies with internships in Biochemistry, Microbiology and Neurobiology.	

## RELEVANT SKILLS

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Languages	<b>German</b>	mother tongue
	<b>English</b>	fluent
	<b>Kiswahili</b>	intermediate
Bioanalytics	Development and implementation of diagnostic bioassays in clinical trial settings. Strong technical background in various lab techniques including advanced problem-solving skills.	
Bioinformatics	Usage of LINUX commands and tools for analysis of next-generation sequencing data. Scientific programming for analysis and visualization of data using the R environment.	
Lab management	Qualified in management of laboratories including quality control, supply chain management and supervision of technicians and students.	

## REFERENCES

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Available upon request

## PUBLICATIONS

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